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The Effect of Adrenaline on Cardiac AMP-Activated Protein Kinase

by

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A thesis submitted for the degree of Doctor of Philosophy
in the University of London

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Abstract

In freshly isolated adult rat cardiac myocytes, adrenaline decreased AMPK activity and Thr172 phosphorylation in AMPK α-subunits. This was associated with a decrease in AMPK-driven phosphorylation of acetyl-CoA carboxylase. The effect of adrenaline on AMPK was rapid with a half-time of approximately 4 minutes. The inactivation of AMPK by adrenaline was not associated with detectable changes in the myocyte contents of ATP, ADP, AMP, creatine, and creatine phosphate. The effect of adrenaline on AMPK was preserved under conditions where AMPK was activated by palmitate or sorbitol, but it was markedly diminished when AMPK was activated by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), oligomycin, or phenformin. The effect of adrenaline was partially blocked by propranolol (β-adrenergic antagonist) or phentolamine (α_1-adrenergic antagonist) while it was essentially abolished when both blockers were present, suggesting involvement of both β and α_1 adrenergic receptors. Isoproterenol (β-adrenergic agonist) and phenylephrine (α_1-adrenergic agonist) could also decrease AMPK activity and Thr172 phosphorylation. Adrenaline did not increase phosphorylation of Ser485/491 in the AMPK α-subunit, but incubation of a catalytically inactive AMPK complex (α1β1γ1) with a cell lysate from adrenaline-treated myocytes increased phosphorylation of the AMPK β1 subunit. The effect of adrenaline was not mimicked by conditions that activated cAMP-pathways and was not blocked by an inhibitor of calcium/calmodulin-dependent kinase II. However, a phorbol ester could mimic the effect of adrenaline on AMPK, suggesting the possible involvement of PKC isoforms.

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Table of Contents

List of Figures and Table

Figures

Table

Abbreviations

8-CPT-2'-O-Me-cAMP	8-(4-chlorophenylthio)-2'-O-methyladenosine 3', 5'-cyclic monophosphate
AC	adenylate cyclase
ACC	acetyl-CoA carboxylase
ACOD	acyl-CoA oxidase
ADP	adenosine 5'-diphosphate
AKAP	A-kinase anchoring ptrotein
AMP	adenosine 5'-monophosphate
AMPK	AMP-activated protein kinase
AMPKK	AMP-activated protein kinase kinase
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AP-2	adaptor protein complex 2
aPKC	atypical protein kinase C
AR	adrenergic receptor
ARF6	ADP-ribosylation factor 6
ASC	Association with SNF1 Complex
ASK1	apoptosis signal-regulated kinase 1
ATM	ataxia telangiectasia mutated
ATP	adenosine 5'-triphosphate
βARK	β adrenergic receptor kinase
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	carboxyl (C)-terminus
C	catalytic subunit
CAMK	calcium/calmodulin-dependent protein kinase
CAMKK	calcium/calmodulin-dependent protein kinase kinase
cAMP	cyclic adenosine monophosphate
CBS	cystathione-β-synthase
CHO	Chinese hamster ovary
cPKC	conventional protein kinase C
Cr	creatine

CrP	creatine phosphate
CTX	cholera toxin
DAG	diacylglycerol
DNP	dinitrophenol
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eEF2	eukaryotic elongation factor 2
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
eNOS	endothelial nitric oxide synthase
Epac	Exchange Protein Directly Activated by cAMP
ERK	extracellular signal-regulated kinase
FABPpm	plasma membrane associated fatty acid binding protein
FAT	fatty acids translocase
GAP	GTPase activator protein
GBD	glycogen binding domain
GDP	guanosine 5'-diphosphate
GEF	guanine nucleotide exchange factor
Glut4	glucose transporter 4
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
GSK3	glycogen synthase kinase 3
GTP	guanosine 5'-triphosphate
Hepes	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
IGF-1	insulin-like growth factor-1
IP_3	inositol-1,4,5-trisphosphate
JAK1	Janus kinase 1
JNK	c-Jun N-terminal kinase
KHB	Krebs Henseleit Bicarbonate buffer

KIS	Kinase Interacting Sequence
Lbc	lymphoblastoma crisis
LCFA	long chain fatty acid
MAGI-1	membrane associated guanylate kinase-like protein inverted-1
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MEF2	myocyte enhancer factor 2
MEHA	3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline
MEK	mitogen-activated protein kinase/extracellular signal-regulated kinase kinase
MEKK	mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase
MCD	malonyl-CoA decarboxylase
MLTK	mixed lineage kinase-like mitogen-activated protein triple kinase
MKK	mitogen-activated protein kinase kinase
MKKK	mitogen-activated protein kinase kinase kinase
MO25α	mouse protein 25 α
mTOR	mammalian target of rapamycin
Myr	myristoylation
MUK	MAPK upstream kinase
N	amino (NH_2)-terminus
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
NFAT	nuclear factor of activated T lymphocytes
NHERFP-1/2	Na^+-H^+ exchanger regulatory factor proteins-1/2
nPKC	novel protein kinase C
NSF	N-ethylmaleimide-sensitive factor
PA	phosphatidic acid
PAK	p21-activated kinase
PC	phosphatidylcholine
PCA	perchloric acid
PDE	phosphodiesterase

PDK1	phosphoinositide-dependent protein kinase 1
PDZ	PSD-95, Discs-large, and ZO-1
PEG	polyethylene glycol
PFK	phosphofructokinase
PH	pleckstrin homology
PI3K	phosphoinositide-3-kinase
PIK	phosphoinositide kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PKI	protein kinase inhibitor
PLA_2	phospholipase A_2
PLB	phospholamban
PLC	phospholipase C
PLD	phospholipase D
PM	plasma membrane
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethanesulfonyl fluoride
PP	protein phosphatase
PS	phosphatidylserine
PSD-95	post synaptic density protein-95
PtdIns	phosphatidylinositol
PtdIns(3,4,5)P_3	phosphatidylinositol-3,4,5-trisphosphate
PtdIns(3,4)P_2	phosphatidylinositol-3,4-bisphosphate
PtdIns(3)P	phosphatidylinositol-3-phosphate
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
PTX	pertussis toxin
PVDF	polyvinyldene difluoride
PYK2	protein tyrosine kinase 2
R	regulatory subunit
RACK	Receptor for Activated C-kinase
RGS	regulators of G-protein signalling
RICK	Receptor for Inactive C-kinase

Rictor	rapamycin-insensitive companion of mTOR
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SH	Src homology
siRNA	small interfering RNA
SNF1	Sucrose non-fermenting 1
SPRK	Src homology 3 domain-containing proline-rich protein kinase
SR	sarcoplasmic reticulum
STRADα	STE20-related adaptor α
TAK1	transforming growth factor β-activated kinase 1
TBS	Tris buffered saline
Tris	tris(hydroxymethyl)-aminomethane
TSC2	tuberous sclerosis complex 2
UCP1	uncoupling protein 1
ZMP	5-aminoimidazole-4-carboxamide ribonucleoside monophosphate

Chapter 1: Introduction

1.1 The physiological roles of cardiac adrenergic receptors

The stimulation of cardiac adrenergic receptors, which occurs following increased sympathetic activity and the increased release of adrenaline from the adrenal medulla (e.g. during exercise and fight-or-flight response), causes an acute increase in the cardiac output as a result of the increased heart rate (positive chronotropic effect), relaxation rate (positive lusitropic effect), and force of contraction (positive inotropic effect). During exercise, the adrenergic stimulation of cardiac output, together with skeletal muscle vasodilation, increases blood flow to the active muscles. The sympathetic activity is also increased during conditions which cause a fall in the arterial blood pressure (e.g. hemorrhage and heart failure) and the adrenergic stimulation of cardiac output is an important part of the hemodynamic defence reaction which attempts to restore the blood pressure. The stimulation of cardiac adrenergic receptors also causes alterations in the cardiac fuel metabolism to meet the increased demand for energy that accompanies the increased contractile function. The heart contractile activity requires a continuous supply of energy in the form of ATP. The heart is an omnivorous organ which can utilise a wide range of substrates including fatty acids, glucose, lactate, ketone bodies and amino acids. However, in the normal-well oxygenated heart, approximately 70% of ATP is generated by the oxidation of fatty acids and the rest mainly comes from carbohydrate (glucose and lactate) oxidation. Adrenergic stimulation causes a rapid burst of glycogenolysis followed by stimulation of exogenous glucose uptake, as well as stimulation of

intracellular triacylglycerol (TAG) turnover and peripheral lipolysis, increasing the fatty acid supply to the heart (Goodwin *et al* 1998 a and b; Collins-Nakai *et al* 1994; Williamson 1964; Gold *et al* 1965; Crass *et al* 1975). During adrenergic stimulation, both fatty acid and glucose oxidation and glycolysis are stimulated, but glucose utilisation is preferentially increased over fatty acid oxidation, increasing the relative contribution of carbohydrate to the overall ATP production (Goodwin *et al* 1998b; Collins-Nakai *et al* 1994). The selective utilisation of glucose is thought to be advantageous in the situations of increased cardiac workload as the ATP yield per oxygen consumed is higher for the oxidation of carbohydrate than that of fatty acids (Goodwin *et al* 1998b).

Plasma catecholamines are elevated in the patients with chronic heart failure (Rundqvist *et al* 1997) and prolonged stimulation of adrenergic receptors is thought to be a factor contributing to the development of pathological hypertrophy. This is supported by numerous studies done with the whole animals or isolated heart/myocytes (Scheuer 1999) and by the observation that transgenic mice lacking endogenous adrenaline and noradrenaline developed less hypertrophy in response to pressure overload by aortic constriction (Esposito *et al* 2002). Cardiac hypertrophy is an adaptive response to increased workload and is characterised by increased protein synthesis and cardiac myocyte size, and in the case of pathological hypertrophy, it is also associated with the expression of genes that are normally expressed in foetal cardiac myocytes, and with structural and metabolic abnormalities including reduced fatty acid oxidation and energy starvation (Sugden and Clerk 1998a; Allard *et al* 2006). Pathological hypertrophy is often associated with myocardial cell death (Dorn II and Brown 1999). This effect may be secondary to the metabolic abnormalities in the hypertrophied heart but it may also be partly due to direct activation of apoptotic signalling by adrenergic receptor stimulation (Krishna Singh *et al* 2001).

1.2 The adrenergic receptors

The adrenergic receptors are a subfamily of the G protein coupled receptors (GPCRs), which trigger intracellular signalling by activating heterotrimeric G proteins upon stimulation. Based on the pharmacological properties and the

downstream signalling pathways they activate, the adrenergic receptors are classified into three types; β, α_1 and α_2, each of which is further divided into several genetically distinct subtypes (β_1, β_2, β_3, α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, and α_{2C}). In addition to these, there are the α_{1L} receptor, which is a putative α_1 subtype (Muramatsu *et al* 1990); four splice variants of α_{1A} ($1_{A\text{-}1}$ to $1_{A\text{-}4}$) (Hirasawa *et al* 1995; Chang *et al* 1998); and a putative 'β_4 receptor' which is probably the β_1 receptor (Brodde *et al* 2001). The cardiac expression of the adrenergic receptor types and subtypes depends on the species and the developmental stage. The heart expresses both β and α_1 receptors. α_2 receptors do not appear to be expressed in the heart in many species including human (Xiang and Kobilka 2003; Brodde *et al* 2001). In the human heart α receptors are much less abundant than β receptors (the ratio of β receptors to α receptors is about ten to one) (Hoffman and Lefkowitz 2002). However, the rat heart expresses relatively high levels of α_1 receptors (Michelotti *et al* 2000). All three α_1 subtypes are expressed in rodent cardiac myocytes with α_{1B} being the most abundant (Yang *et al* 1998; Price *et al* 1994a; Wolff *et al* 1998). In contrast, the mRNA for α_{1A} (1A-4 and 1A-1) predominates in the human heart (Chang *et al* 1998; Price *et al* 1994b). In both rodents and human, α_{1D} is minimally expressed in the heart (Tanoue *et al* 2002; Yang *et al* 1999, Price *et al* 1994a). Both β_1 and β_2 receptors are expressed in the rat (Morisco *et al* 2001b) and human heart (Brodde *et al* 2001). In both species, β_1 is more abundant than β_2 in the adult heart (Brodde *et al* 2001; Morisco *et al* 2001b). However, the opposite pattern is seen in the neonatal rat heart (Morisco *et al* 2001b). The β_3 receptor is also expressed in the human heart (Gauthier *et al* 1996 and 1998).

All adrenergic receptors have similar overall structures which are characterised by seven transmembrane helices linked by three intracellular and three extracellular loops (Strosberg *et al* 1993; Graham *et al* 1996; Yarden *et al* 1986). The N-terminal region, which contains a number of N-linked glycosylation sites, is located at the extracellular side of the plasma membrane while the C-terminal tail is cytosolic. Mutagenesis and chimeric receptor studies have shown that the amino acid sequence at the intracellular loop connecting the 5^{th} and 6^{th} transmembrane regions is important for the interaction of the receptor with G proteins and for the specificity of the receptor - G protein coupling (Wu *et al* 1995; Xiao 2001). The C-terminal tail has a number of important functions. It contains phosphorylation sites for PKA, PKC, and GRK which are involved in the desensitisation of the adrenergic receptors

(Garcia-Sainz *et al* 2000). The β_2 receptor can be palmitoylated at a C-terminal cysteine (O'Dowd *et al* 1989). The palmitoylation at this site may regulate the desensitisation by inhibiting PKA phosphorylation of the receptor (Moffett *et al* 1996) or it may be involved in the association of hydrophobic proteins with the receptor (Morris and Malbon 1999). The C-terminal tail of the β receptor also contains a PDZ domain-binding motif (Hall and Lefkowitz 2002). Several PDZ-domain-containing proteins including PSD-95 and MAGI-2 have been shown to associate with the β_1 receptor (Hall and Lefkowitz 2002). The β_2 receptor associates with NHERFP-1 and NHERFP-2 through PDZ domain (Hall and Lefkowitz 2002). However, the role of the PDZ domain-binding motif in cardiac adrenergic signalling is not clear. The C-terminus of the β_2 receptor also interacts with the A-kinase anchoring proteins (Section 1.5.1) such as AKAP220 (gravin) (Fan *et al* 2001) and AKAP79 (Cong *et al* 2001).

1.3 GTP-binding protein (G protein) signalling

Adrenoreceptor stimulation causes the activation of G protein signalling through the following sequence of events.

1) In the absence of agonists, the adrenoreceptors resonate between the basal state (R) and the activated state (R*). Only R* can interact with G protein and activate downstream signalling. This concept of spontaneous activation is supported by the observation that overexpression of some adrenoreceptors (e.g. β_2) (Bond *et al* 1995) can activate downstream pathway in the absence of agonist. However this does not appear to be the universal property seen in all adrenoreceptor subtypes as over-expression of the β_1 receptor does not activate downstream pathway in the absence of the agonist (Zhou *et al* 2000). The agonist preferentially binds to R* and locks the receptor in the activated state, while the antagonist binds to both R and R* with similar affinity and blocks agonist binding. A negative agonist preferentially binds to R and locks the receptor in the inactive state. In addition to this simplified scheme, the existence of multiple R* states, which are coupled to different downstream signalling events have been suggested for α_{1B} (Perez *et al* 1996) and β2 receptors

(Peleg *et al* 2001; Ghanouni *et al* 2001). Adrenergic receptors may also dimerise upon activation (Hebert and Bouvier 1998).

2) The heterotrimeric G proteins are composed of the G_α subunit, which is bound to GDP in the basal state, G_β, and G_γ. G_β and G_γ are tightly associated. The activated receptor acts as a guanine nucleotide exchange factor (GEF) for the heterotrimeric G proteins and stimulates the exchange of GDP bound to G_α for GTP. The GTP binding lowers the affinity of G_α to $G_{\beta\gamma}$ and the receptor, resulting in the dissociation of the active G_α from the complex and the release of $G_{\beta\gamma}$ dimer. Both G_α and $G_{\beta\gamma}$ then interact and modulate a number of downstream signalling components.

3) Hydrolysis of GTP by the intrinsic GTPase activity of G_α causes re-association of G_α with $G_{\beta\gamma}$, terminating G_α and $G_{\beta\gamma}$ signalling.

There are four classes of G_α (G_s, G_i, G_q, and $G_{12/13}$) which activate distinct signalling pathways (Morris and Malbon 1999; Eschenhagen 1993). The G_s class includes G_s, which is widely expressed including the heart, and G_{olf}, which is only found in the brain and olfactory epithelium. The G_s class of G proteins activates adenylate cyclase (AC), which catalyses the formation of the second messenger cAMP from Mg^{2+}ATP. cAMP activates PKA which mediates many biological effects of adrenergic receptor stimulation. There are nine classes of AC with splice variants (Houslay and Milligan 1997). They are all activated by G_s but they differ in the regulation by other factors such as Ca^{2+}, PKC and PKA. The predominant isoforms expressed in the heart are ACV and VI (Espinasse *et al* 1995). ACV and VI are phosphorylated and inhibited by PKA (Iwami *et al* 1995; Chen *et al* 1997). This may serve as a negative feedback mechanism for cAMP and PKA signalling (Bauman *et al* 2006). PKC has different effects on ACV and ACVI; it inhibits ACVI (Lai *et al* 1997) but it activates ACV (Kawabe *et al* 1994). ACV and VI have been reported to be insensitive to Ca^{2+}/calmodulin (Tanssig and Gilman 1985; Katsushita *et al* 1992), although the study by Cooper *et al* suggests ACV and VI are inhibited by Ca^{2+} (Cooper *et al* 1994). Apart from activating AC, G_s has been suggested to directly modulate the activity of the L-type Ca^{2+} channel (Section 1.5.3) and Na^+ channel (Schubert *et al* 1989).

The G_i class includes G_{i-1}, G_{i-2}, G_{i-3}, G_{oA}, G_{oB}, G_{t1}, G_{t2}, and G_z. G_{i-1}, G_{i-2}, and G_{i-3} are widely expressed but the heart does not express G_{i-1} (Jones and Reed 1987). The predominant G_i expressed in the heart is G_{i-2} (Bohm *et al* 1994). G_o and G_t are found in the brain and retina, but a low level of G_o mRNA is also found in the human and rat heart (Eschenhagen *et al* 1993). G_i is inhibitory to ACV/VI (Taussig *et al* 1994; Ran *et al* 2003).

The G_q class includes G_q, G_{11}, G_{14}, G_{15}, and G_{16}. G_q and G_{11} are ubiquitously expressed. All members of the G_q class activate phospholipase Cβ (PLCβ) (Wu *et al* 1992). There are four isoforms of PLCβ (PLCβ1-4). The mRNA for PLCβ1 and 3 are found in the heart (Schnabel *et al* 1996 and 2000). PLC hydrolyses phosphatidylinositol-4,5-bisphopsphate (PtdIns(4,5)P_2) to generate diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 causes Ca^{2+} release from the SR through the IP_3 receptor (Section 1.5.3). DAG is an activator of conventional and novel PKC isoforms (Section 1.5.7).

The $G_{12/13}$ class is widely expressed, but their function is not well understood. The suggested downstream signalling of these G proteins includes Rho and PYK2 (Kurose 2003).

In addition to differences in their downstream pathways, these four classes of G proteins show differences in their susceptibility to ADP-ribosylation by cholera and pertussis toxins and this property is useful for identifying which class of G protein is involved in a given cellular response. G_s proteins are ADP-ribosylated by cholera toxin (CTX), which inhibits the intrinsic GTPase activity. This causes the constitutive activation of AC and cAMP generation. The members of the G_i family, except for G_z, are irreversibly ADP-ribosylated by pertussis toxin (PTX) which uncouples the G protein from the receptor and inhibits signalling through these G proteins. The members of G_q and $G_{12/13}$ families are insensitive to both CTX and PTX.

In addition to the four classes of G proteins described above, a G protein with an unusually high molecular weight (78 kDa) termed G_h has been isolated from the bovine heart (Baek *et al* 1993). This G protein has been shown to associate with the

α_1 receptor and PLC and mediate the activation of PLC by α_1 agonists *in vitro* (Das *et al* 1993; Baek *et al* 1993).

Like G_α, $G_{\beta\gamma}$ regulates activity of a number of effectors including class IB PI3K (Section 1.5.5), AC isoforms (Taussig and Gilman 1995), and PLCβ (Smrcka and Sternweis 1993). The effect of $G_{\beta\gamma}$ on ACV/VI is controversial. Bayewitch *et al* have reported that ACV and VI are inhibited by $G_{\beta\gamma}$ (Bayewitch *et al* 1998), although others found no effect of $G_{\beta\gamma}$ on ACV and VI (Taussig and Gilman 1995). In contrast, Gao *et al* have recently reported that $G_{\beta\gamma}$ enhances activity of ACV and VI in the presence of $G_{\alpha s}$ or forskolin (Gao *et al* 2006). PLCβ isoforms, particularly PLCβ2 and 3 are activated by $G_{\beta\gamma}$ (Park *et al* 1993). $G_{\beta\gamma}$ also plays a role in desensitisation by recruiting βARK to the membrane (Pitcher *et al* 1995).

Both β_1 and β_2 receptors are coupled to G_s and the stimulation of both receptors cause activation of AC and cAMP accumulation (Chesley *et al* 2000; Bartel *et al* 2003). Several lines of evidence suggest that the β_2 receptor is additionally coupled to G_i. Some downstream effects of selective β_2 stimulation, such as activation of PI3K (Chesley *et al* 2000) and PLA_2 (Pavoine *et al* 1999), are PTX sensitive. PTX has also been shown to enhance the stimulation of contraction by the β_2 receptor (Xiao *et al* 1995). Lastly, in mouse cardiac myocytes, association of the β_2 receptor and G_i (G_{i-2} and G_{i-3}) has been demonstrated by photoaffinity labelling and by immunoprecepitation using a G_i specific antibody (Xiao *et al* 1999). Like the β_2 receptor, the β_3 receptor also appears to dually couple to both G_s and G_i (Section 1.4.1).

The α_1 receptors are coupled to multiple G proteins. A transient transfection experiment with COS-7 cells showed that all three α_1 subtypes can couple to G_q and G_{11} to activate PLCβ1 in response to noradrenaline (Wu *et al* 1992). However, the three subtypes showed a difference in the coupling to G_{14} and G_{16}; α_{1B} coupled to both G_{14} and G_{16}, α_{1A} only coupled to G_{14}, and α_{1D} did not couple to G_{14} or G_{16} (Wu *et al* 1992). The four splice variants of α_{1A} all couple to the PLC-Ca^{2+} pathway (Hirasawa *et al* 1995; Chang *et al* 1998). The heart α_1 receptor may also associate with G_h (Baek *et al* 1993). Some downstream effects of cardiac α_1 stimulation are PTX sensitive (Steinberg *et al* 1985) or blocked by inhibitors of $G_{12/13}$ (Maruyama *et*

al 2002), indicating G_i and $G_{12/13}$ are also downstream of α_1 receptors. Gallego *et al* observed that stimulation of the cardiac α_1 receptor (α_{1A} and α_{1B}) caused a β receptor-independent (but G_s- and AC-dependent) increase in cAMP and PKA activity (Gallego *et al* 2005). This effect was only seen in intact cells, and the effect was inhibited by disrupting the cytoskeleton by colchicine, indicating compartmentalisation of this effect (Gallego *et al* 2005). It was also demonstrated that phenylephrine increased physical association of G_s with the α_1 receptor (Gallego *et al* 2005).

G protein signalling is regulated by a number of mechanisms. Prolonged stimulation of adrenergic receptors reduces the responsiveness of the receptors to activate downstream signalling pathways by the process termed desensitisation. This process involves phosphorylation of the receptors by PKA, PKC and a group of kinases called the G protein receptor kinases (GRKs) (Benovic *et al* 1985; Diviani *et al* 1996 and 1997; Guimond *et al* 2005; Lattion *et al* 1994). Phosphorylation of adrenergic receptors by PKA, PKC, and GRKs inhibits interaction of the receptors with G proteins and inhibits stimulation of G protein signalling (Benovic *et al* 1985; Diviani *et al* 1996, Guimond et al 2005). The phosphorylation by GRKs causes agonist-specific (homologous) desensitisation, while PKA and PKC phosphorylations cause non-agonist specific (heterologous) desensitisation (Lattion *et al* 1994). Several isoforms of GRK are expressed in the rat heart including GRK2 (also known as β adrenergic receptor kinase (βARK)), 3, 5, and 6 (Inglese *et al* 1993). Studies with mice over-expressing different GRKs suggested that distinct GRKs may be involved in desensitisation of the different adrenoreceptor subtypes *in vivo*. The α_{1B} receptor appears to be phosphorylated mainly by GRK3 *in vivo* (Eckhart *et al* 2000). However, the over-expression of GRK3 did not affect β signalling (Eckhart *et al* 2000), which was attenuated by over-expression of GRK5 (Rockman *et al* 1996). In addition to inhibiting G protein coupling, the phosphorylation of the receptors by GRKs and possibly PKC (Fonseca *et al* 1995) also leads to the internalisation of the receptors by recruiting β-arrestin, which binds a number of proteins involved in endocytosis including clathrin, clathrin adaptor AP-2, NSF, and ARF6 (Laporte *et al* 1999 and 2000; McDonald *et al* 1999; Claing *et al* 2001).

Another key regulatory step in G protein signalling is the hydrolysis of GTP bound to the active G_α by the intrinsic GTPase activity of G_α. This process is accelerated by the GTPase activator proteins (GAPs) which, by doing so, negatively regulate G protein signalling. PLCβ appears to act as a GAP for G_q (Berstein *et al* 1992). A class of GAPs called the regulators of G-protein signalling (RGS) are a family of proteins that share a conserved RGS domain that can accelerate the GTPase activity of G_α (Wieland and Mittmann 2003). In the mammalian heart, at least 13 members of the RGS protein family are expressed including the most-well studied RGS4 (Wieland and Mittmann 2003). The majority of RGS proteins identified to date, including RGS4, enhance the GTPase activity of $G_{i/o}$ and $G_{q/11}$ and inhibit signalling downstream of these G proteins (Wieland and Mittmann 2003). Some RGS proteins including RGS3, which is expressed in the heart, also inhibit signalling mediated by $G_{\beta\gamma}$ by binding and sequestering these subunits (Shi *et al* 2001). GRK2 contains a domain similar to the RGS domain which binds $G_{q/11}$ and it seems to regulate G_q signalling by sequestering the activated G_q (Kozasa 2001). RGS-PX1 is another protein containing a RGS-like domain. This protein acts as a GAP for G_s and inhibits isoproterenol-induced AC activation in neonatal rat cardiomyocytes (Zheng *et al* 2001). Another RGS-like domain-containing protein p115-Rho-GEF is a specific GAP for G_{12} and G_{13} (Kozasa *et al* 2001).

G protein signalling may also be regulated by direct post-translational modifications of the G proteins. The palmitoylation of G_α has been shown to protect G_z and G_{i-1} from GAP activity and RGS4, respectively (Tu *et al* 1997). The phosphorylation of G_{i-2} by PKC may block the effect of G_{i-2} to inhibit AC (Bushfield *et al* 1990; Strassheim and Malbon 1994). $G_{q/11}$ has been reported to be tyrosine phosphorylated by an unidentified tyrosine kinase upon receptor activation and this phosphorylation promotes the interaction of $G_{q/11}$ with the receptor (Umemori *et al* 1997).

1.4. An overview of cardiac adrenergic signalling

This section briefly outlines signalling pathways involved in the different biological effects of cardiac adrenergic receptor stimulation.

1.4.1 Contraction

The adrenergic stimulation of contractile function is predominantly regulated by the β_1 receptor through an increase in cAMP and activation of PKA, which phosphorylates key proteins involved in Ca^{2+} homeostasis and contraction including the L-type Ca^{2+} channel, the ryanodine receptor, phospholamban (PLB), and troponin I and C proteins. Phosphorylation of the L-type Ca^{2+} channel increases Ca^{2+} influx across the sarcolemma which triggers Ca^{2+}-induced Ca^{2+} release from the SR through the ryanodine receptor, leading to an increase in the cytosolic Ca^{2+} and contractile amplitude. PKA may also contribute to this process by increasing the open probability of the ryanodine receptor (Bers 2002). The lusitropic effect is due to a PKA phosphorylation of PLB (at PKA site, Ser16) and troponin I which accelerates Ca^{2+} sequestration into the SR and reduces the sensitivity of the myofilament for Ca^{2+}, respectively. Some of these PKA targets are also phosphorylated by CAMKII. CAMKII has been shown to increase the Ca^{2+} current through the L-type Ca^{2+} channel and the relaxation rate through the phosphorylation of PLB at the CAMKII site, Thr17 (Wang *et al* 2004). While the cAMP level declines quickly after adrenergic stimulation, CAMKII activity is sustained for longer (Wang *et al* 2004). Thus, CAMKII has been suggested to be the primary regulator of excitation-contraction coupling during prolonged β_1 stimulation (Wang *et al* 2004).

Stimulation of the β_2 receptor also causes an increase in cAMP through the activation of G_s. However, unlike stimulation of the β_1 receptor, which causes a global increase in cAMP and leads to PKA phosphorylation of membrane targets (e.g. Ca^{2+} channel) as well as intracellular targets (e.g. PLB), stimulation of the β_2 receptor in adult rat cardiac myocytes has been shown to cause a compartmentalised increase in cAMP which increases PKA phosphorylation of the L-type Ca^{2+} channel without affecting PLB phosphorylation (Jo *et al* 2000). Consistent with the lack of phosphorylation of PLB, the β_2 receptor stimulation increased the Ca^{2+} transient and contraction amplitude without increasing the relaxation rate in these myocytes (Jo *et al* 2002). The exact mechanism responsible for the compartmentalisation of the β_2 cAMP signalling is unknown, but the activation of G_i and PI3K, which is downstream of G_i, are essential for this effect (Jo *et al* 2000). Both the β_2 receptor and G_i are primarily

found in calveoli (Rybin *et al* 2000), suggesting the cAMP accumulation by the β_2 receptor is confined to the calveoli. However this compartmentalised cAMP signalling apparently does not exist in human cardiac myocytes which showed phosphorylation of PLB by PKA after β_2 stimulation (Kaumann *et al* 1996). In contrast to rat and human cardiac myocytes, stimulation of the mouse β_2 receptor does not cause an inotropic response despite a dual coupling of the β_2 receptor with G_s and G_i (Xiao *et al* 1999). Thus, in mouse cardiac myocytes, a β_2-mediated increase in cAMP appears to be completely negated by simultaneous activation of G_i.

The signalling of the cardiac β_3 receptor and its role in contractile activity and other processes are not well-understood. However several studies have demonstrated that selective stimulation of the cardiac β_3 receptor causes a small decrease in contractility (Devic *et al* 2001; Gauthier *et al* 1996 and 1998). This effect is attributed to the G_i-dependent activation of nitric oxide synthase (Gauthier *et al* 1996). The β_3 receptor also appears to be coupled to the G_s-cAMP pathway because selective stimulation of the β_3 receptor in the presence of PTX increased contractility, although this effect would be masked by the inhibitory effect through G_i during the normal stimulation of the receptor (Devic *et al* 2001). The β_3 regulation of contraction may play a role in the deterioration of heart contractile activity during chronic adrenergic stimulation because, unlike β_1 and β_2 receptors, the β_3 receptor lacks the recognition motif for βARK and PKA which is involved in desensitisation (Strosberg *et al* 1993).

In addition to β_1 receptors, α_1 receptors also contribute to contractile activity by increasing Ca^{2+} and activating PKC, which has been implicated in the regulation of Ca^{2+} homeostasis (Kamp *et al* 2000), in modulation of properties of the contractile proteins (Takeishi *et al* 1998), and in ionic balance (Gambassi *et al* 1998 and 1992). However, α_1 receptors are generally considered to play a minor role in the stimulation of contractile activity. The effect of α_1 receptors on contractile function also appears to depend on the species and the developmental stage (Tanoue *et al* 2003).

1.4.2 Fuel catabolism

Adrenergic receptor stimulation increases fuel catabolism *via* both α_1 and β receptors. Key regulators of this process are PKA and Ca^{2+}. PKA phosphorylates and activates key enzymes in catabolism including phosphorylase kinase (Hayes and Mayer 1981), phosphofructokinase 2 (PFK2) (Depre *et al* 1998) and TAG lipase (Small *et al* 1989). PKA has also been shown to phosphorylate and inactivate the cardiac isoform of acetyl-CoA carboxylase (ACC) (ACC2/ACC280) *in vitro* (Boone *et al* 1999). ACC2 plays an important role in the regulation of the β-oxidation of long chain fatty acids in muscles by synthesising malonyl-CoA, a metabolite which inhibits the translocation of long chain fatty acids into the mitochondria for β-oxidation by allosterically inhibiting carnitine palmitoyltransferase 1 (Kerner and Hoppel 2000). A decrease in malonyl-CoA has been observed during adrenergic stimulation, consistent with a stimulation of fatty acid oxidation (Goodwin and Taegtmeyer 1999). However, whether ACC2 is inactivated by PKA *in vivo* is not clear. Although Boone *et al* observed phosphorylation of ACC2 in cardiac myocytes in response to isoproterenol, partially purified ACC from isoproterenol-treated cells did not show a change in catalytic activity (Boone *et al* 1999). Goodwin and Taegtmeyer also did not observe any change in ACC activity despite a decrease in malonyl-CoA during adrenergic stimulation in the perfused heart (Goodwin and Taegtmeyer 1999). These investigators attributed the decrease in malonyl CoA to the activation of malonyl-CoA decarboxylase (MCD) (Goodwin and Taegtmeyer 1999). However, how MCD is activated during adrenergic stimulation was not investigated in this study. The increase in Ca^{2+} which occurs after β receptor or α_1 receptor stimulation activates a number of metabolic enzymes including phosphorylase kinase, pyruvate dehydrogenase (through pyruvate dehydrogenase phosphatase), NAD^+-isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase (Werth *et al* 1982; McCormack and Denton 1979; Denton *et al* 1978; McCormack and England 1983; Denton *et al* 1996). The α_1 receptor has been reported to activate PFK in perfused hearts by Ca^{2+}-dependent process, although PFK was not directly activated by Ca^{2+} (Patten *et al* 1982). The α_1 receptor may also contribute to the activation of mitochondrial enzymes by activating the mitochondrial Ca^{2+} uniporter (Kesser and Crompton 1981). The mechanism of the adrenergic stimulation of glucose uptake is

not completely understood. Both α_1 and β receptors are likely to be involved in the stimulation of Glut4 translocation and glucose uptake (Rattigan *et al* 1986; Fischer *et al* 1996a and 1996b; Clark and Patten 1984). The proposed downstream pathways for both receptors include Ca^{2+} (Clark and Patten 1984; Rattigan *et al* 1986) and PI3K/PKB (Doenst and Taegtmeyer 1999; Morisco *et al* 2005). PKC has also been implicated in the stimulation of glucose and fatty acid uptake in the heart (Luiken *et al* 2004).

1.4.3 Hypertrophy

The role of the α_1 receptor and G_q signalling in the development of hypertrophy is well-documented (reviewed by Dorn II and Brown 1999). Although the signalling pathways downstream of G_q are complex and incompletely understood, PKC and MAPKs (ERK, p38, and JNK) have been identified as key regulators of G_q-mediated hypertrophy (Dorn II and Force 2005; Michel *et al* 2001; Steinberg 2002). MAPKs regulate a wide range of transcription factors (Michel *et al* 2001). PKC regulates activities of transcription factors such NFATc3 (through inhibition of GSK3) (Dorn II and Force 2005), class II histone deacetylase (Vega *et al* 2004), and MAPKs (Section 1.5.9). There has been interest in the identification of the α_1 receptor subtypes responsible for hypertrophic growth. Although α_{1A}, α_{1B}, and α_{1D}, all couple to G_q, a number of studies suggest that the signalling by these subtypes are distinct. Transfection experiments using PC12 cells showed the three subtypes display differences in the efficiency of inositol phosphate production and Ca^{2+} release, with efficacy in the order; $\alpha_{1A}>\alpha_{1B}>\alpha_{1D}$ (Zhong and Minneman 1999). In the same experiments, the three subtypes also showed differences in the coupling to MAPKs. While ERK, JNK, and P38 were all activated by α_{1A}, α_{1B} only activated ERK and P38 (Zhong and Minneman 1999). The α_{1D} receptor did not cause significant activation of MAPKs (Zhong and Minneman 1999). In the rat heart, phospholipase D (PLD), the enzyme though to be important for the sustained activity of PKC (Section 1.5.7), was activated by α_{1A} and α_{1D} receptors, but not by α_{1B} (Mier *et al* 2002). The three subtypes also show distinct subcellular localisations; α_{1A} is localised at both the plasma and intracellular membranes; α_{1B} is localised primarily at the plasma membrane; and α_{1D} is confined to intracellular membranes (Plascik and Perez 2001).

Of the three subtypes, α_{1D} is generally not thought to play a major role in cardiac adrenergic signalling due to its low abundance and poor coupling to downstream signalling pathways. In neonatal cardiac myocytes, the selective activation of α_{1A} has been shown to be sufficient to cause hypertrophy (Autelitano and Woodcock 1998). There is a controversy over the relative importance of the α_{1B} receptor in hypertrophy. Transgenic mice over-expressing constitutively active α_{1B} developed cardiac hypertrophy (Milano *et al* 1994b), but over-expression of the wild type α_{1B} was found to be insufficient to cause hypertrophy (Akhter *et al* 1997). Moreover, in neonatal rat cardiac myocytes, the phenylephrine-induced increase in protein synthesis was inhibited by an α_{1A} antagonist but it was potentiated by α_{1B} antagonists, suggesting that α_{1B} signalling may inhibit hypertrophic signalling by the α_{1A} receptor (Deng *et al* 1998).

In addition to the α_1 receptors, stimulation of the β receptors by the general β agonist isoproterenol has also been demonstrated to cause hypertrophy *in vitro* and *in vivo* (Stein *et al* 1996; Morisco *et al* 2001b). This effect seems to be mediated primarily *via* the β_1 receptor as the stimulation of hypertrophy by isoproterenol was unaffected by β_2 antagonists and over-expression of the β_2 receptor did not cause hypertrophy in mice (Morisco *et al* 2001b; Milano *et al* 1994; Liggett *et al* 2000). The β_1 and α_1 receptors appear to promote hypertrophy *via* distinct pathways. The β_1 effect is primarily mediated by the Ca^{2+}-activated phosphatase calcineurin (PP2B), which increases nuclear translocation of NFAT, by CAMKII, which regulates MEF2 through class II histone deacetylase, and by PKB, which regulates transcription factors such as GATA4 and NFAT3 (Morisco *et al* 2000; Morisco *et al* 2001a; Sucharov *et al* 2006). PKB is also an activator of mTOR signalling and protein translation (Proud 2004).

1.4.4 Apoptosis

The stimulation of apoptosis by prolonged adrenergic stimulation appears to be mediated mainly by the β_1 receptor (Zhu *et al* 2003). The pathways linking the β_1 receptor to apoptosis is not well-understood. The involvement of PKA has been demonstrated by some investigators (Iwai-Kanai et al 1999), but this is disputed by

Zhu *et al* who showed that β_1 receptor-mediated apoptosis was insensitive to specific PKA inhibitors but was blocked by chelating Ca^{2+} or by inhibiting CAMKII (Zhu *et al* 2003). The involvement of calcineurin in β receptor-mediated apoptosis has also been reported (Saito *et al* 2000). Stimulation of the β_2 receptor has been reported to protect cardiac myocytes from apoptosis caused by the β_1 stimulation or H_2O_2 (Chesley *et al* 2000; Zhu *et al* 2001). The anti-apoptotic effect of the β_2 receptor was attributed to the β_2 specific activation of G_i/PI3K/PKB pathway (Chesley *et al* 2000; Zhu *et al* 2001). However a later study showed that PI3K was activated as effectively by the β_1 receptor (Leblais *et al* 2004), suggesting that some other factors are involved in this effect or that different isoforms of PI3K are activated by the β_1 and β_2 receptors. Although over-expression of G_q in the heart causes apoptosis in mice (Adams *et al* 1998), in isolated myocytes the α_1 receptor has been reported to have either no effect on apoptosis (Communal *et al* 1998) or even to have an anti-apoptotic effect (Iwai-Kanai *et al* 1999). Although MAPKs are known as regulators of apoptosis, the precise roles of MAPKs in the adrenergic regulation of apoptosis in cardiac myocytes are not well-understood (Bishopric *et al*; Krishna Singh *et al* 2001). However, the observations that the anti-apoptotic effect of the α_1 receptor was blocked by ERK inhibition (Iwai-Kanai *et al* 1999) and that β receptor-mediated apoptosis was potentiated by p38 inhibition (Communal *et al* 2000) have suggested anti-apoptotic roles of ERK and p38 in cardiac myocytes.

1.5 Signalling components

1.5.1 Protein kinase A (PKA)

At the unstimulated level of cAMP, PKA exists as a holoenzyme consisting of two catalytic (C) and two regulatory (R) subunits. The C subunit is a 40 kDa Ser/Thr kinase that transfers γ phosphate of ATP to substrates containing -RRXSX- consensus sequence. Three isoforms of the C subunit encoded by different genes have been characterised; Cα, Cβ, and Cγ (Francis and Corbin 1994). Cα and Cβ show a high sequence similarity and are ubiquitously expressed while Cγ shows less similarity and is only expressed in the testis (Francis and Corbin 1994). In the holoenzyme, the C subunits are inactivated by interaction with the autoinhibitory

region of R subunits. Each of the R subunits has two cAMP binding sites, and the binding of cAMP to these sites decreases the affinity of the R subunit for the C subunit, releasing the free R subunits and the active C subunits. Four isoforms of the R subunit have been identified and they are divided into two types; RI (43 KDa) and RII (25 kDa) (Francis and Corbin 1994). RIα and RIIα are the predominant isoforms and are ubiquitously expressed, while RIβ and RIIβ are expressed in the central nervous system and reproductive tissue (Francis and Corbin 1994). The R subunits form homodimers through the amino terminal dimerisation domain. An important difference between RI and RII is that RI is predominantly found in cytosol whereas RII is associated with the membrane (Scott 1991). This difference in the subcellular localisation is attributed to the preferential interaction of RII with the A-kinase anchoring proteins (AKAPs). Apart from regulation by the R subunits and cAMP, PKA activity is known to be regulated by several other factors including the nuclear protein kinase inhibitors (PKIs) α, β, and γ, which bind to PKA and inhibit its activity (Koppernd *et al* 2003). PKIs also assist nuclear export of the C subunit (Koppernd *et al* 2003). The C subunit can be inactivated by glutathionylation of Cys 199 which may occur during oxidative stress (Humphries *et al* 2002). .

AKAPs play a key role in PKA signalling by giving specificity to the very broad action of PKA (Ruchr *et al* 2004). PKA is tethered to discrete subcellular locations, usually its targets, by interaction with different AKAPs containing unique subcellular targeting domains (Ruchr *et al* 2004). For example, AKAP15/18, Yotiao, and AKAP79/150 tether PKA to L-type Ca^{2+} channel, potassium channel KCNQ1Ik, and ACV/VI, respectively (Marx *et al* 2002; Fraser *et al* 1998; Gray *et al* 1997; Bauman *et al* 2006). Most AKAPs bind the RII homodimer by interaction of the amphipathic helix with the amino terminal domain of RII (Dodge Kafka *et al* 2006). As well as anchoring PKA to its targets, AKAPs also act as signalling processing units by binding other signalling enzymes such as phosphodiesterases (PDEs) and phosphatases. Localised clustering of PDEs and PKA allows differential control of PKA activity in different subcellular locations. Similarly, clustering of phosphatases and PKA targets by AKAP allows localised control of the duration of the effect initiated by PKA. The activity of PDEs or phosphatases in a given AKAP complex may be regulated by AKAP, PKA or some other proteins present in the complex. For example, AKAP220, which binds PKA and PP1, is a competitive inhibitor for PP1

(Schillace *et al* 2001). Muscle-selective AKAP (mAKAP), which is found at the nuclear envelope and SR of cardiac myocytes forms a complex with a number of proteins including PKA, ryanodine receptor, PDE4D3, PP1, PP2A, PP2B, nesprin-1α, Epac, MEK5 and ERK5 (Kapiloff *et al* 2001; Marx *et al* 2000; Dodge *et al* 2001; Dodge Kafka *et al* 2005). PDE4D3 is phosphorylated at two sites by PKA, resulting in increased activity and increased binding affinity for mAKAP (Sette *et al* 1996; Carlisle Michel *et al* 2004). PDE4D3 is also under control of ERK5 which phosphorylates and inhibits it (Dodge Kafka *et al* 2005). In addition to providing a scaffold for assembly of the PKA signalling complex, some AKAPs also play a more direct signalling role. For example, AKAP-lymphoblastoma crisis (Lbc), which binds PKA and $G\alpha_{12}$, is a Rho selective guanine nucleotide exchange factor (Diviani *et al* 2001).

1.5.2 Exchange Protein Directly Activated by cAMP (Epac)

Epac is a relatively recently discovered downstream effector of cAMP (de Rooji *et al* 1998). It is the broadly expressed cAMP-sensitive guanine nucleotide exchange factor for Rap1 and Rap2, which are small Ras-like GTPases (Kitayama *et al* 1989). The sequence of the putative cAMP binding site of Epac is similar to the cAMP binding site of the regulatory subunits of PKA (de Rooji *et al* 1998). The binding of cAMP relieves the inhibition of the guanine nucleotide exchange factor activity by the cAMP-binding domain. (de Rooji *et al* 1998). The downstream effects of cAMP mediated by Epac can be discriminated from PKA-mediated effects by the use of cAMP analogues which selectively activate Epac (Koppernd *et al* 2003). The role of Epac in the heart is still not well-understood. A recent study suggested Epac may activate Ca^{2+} and Rho signalling in neonatal cardiac myocytes (Morel *et al* 2005).

1.5.3 Ca^{2+}

Ca^{2+} is a common signalling component for both α_1 and β receptor signalling. In the beating heart or electrically paced cardiac myocytes, the β receptor-mediated increase in intracellular Ca^{2+} is attributable to phosphorylation of the L-type Ca^{2+} channel at the sarcolemma by PKA, which increases the mean channel open time

and/or opening probability (van der Heyden *et al* 2005). The L-type Ca^{2+} channel has also been reported to be activated directly by $G_{\alpha S}$ (Lader *et al* 1998; Hamilton *et al* 1991; Imoto *et al* 1988). Because the opening of the L-type Ca^{2+} channel is regulated by the action potential, β receptor stimulation in quiescent cardiac myocytes does not cause a rapid increase in intracellular Ca^{2+}, as seen for the contracting heart or for contracting myocytes (Saini *et al* 2006). However, it appears that the β receptor stimulation still causes a slow accumulation of Ca^{2+} through the L-type Ca^{2+} channel even in the absence of the action potential, since prolonged β_1 stimulation increased Ca^{2+} in quiescent cardiac myocytes and this effect was inhibited by nifedipine (Zhu *et al* 2003). The α_1 receptor-mediated increase in Ca^{2+} is due to production of IP_3 which releases Ca^{2+} from the SR through the IP_3 receptor. α_1 stimulation has also been reported to increase Ca^{2+} influx through the L-type Ca^{2+} channel, but this effect is controversial (Liu *et al* 1994; Gaughan *et al* 1998).

1.5.4 Calcium/calmodulin-dependent protein kinase II (CAMKII)

Of the three CAMK types (CAMKI, II, and IV), which are all activated by Ca^{2+}/calmodulin, CAMKII is most abundant in the heart and most well-studied (Colomer *et al* 2003). The heart also expresses CAMKI but its function is unclear. CAMKIV is only present in low abundance (Colomer *et al* 2003). CAMKII is a homo or heteromultimer of 8-12 CAMKII monomers bound to each other by the C-terminal domain (Anderson 2005). The binding of Ca^{2+}/calmodulin to the N-terminal regulatory region releases the inhibition of the catalytic activity by the pseudosubstrate sequence. Once a CAMKII monomer is activated it phosphorylates adjacent monomers in the holoenzyme at Thr287 (Anderson 2005). This autophosphorylation has two effects; it increases the affinity of the monomer for Ca^{2+}/calmodulin, and it also makes CAMKII active even in the absence of bound Ca^{2+}/clamodulin after the return of the cellular Ca^{2+} to the basal level (Anderson 2005). There are four isoforms of CAMKII encoded by separate genes; α, β, γ, and δ, with splice variants for each isoform (Mayer *et al* 1995). In the heart the predominant CAMKII isoform is CAMKδ, which is further divided into two splice variants, δC (δ2) and δB (δ3) (Edman and Shulman 1994). CAMKδB, which is the predominant isoform, is found in the nucleus whereas CAMKδC is cytosolic (Edman and

Shulman 1994; Zhu *et al* 2003). CAMKδB and CAMKδC appear to have distinct roles; CAMKδC is implicated in the regulation of contraction and apoptosis while δB is implicated in hypertrophy (Zhang *et al* 2002; Maier *et al* 2003; Zhu *et al* 2003).

β_1 receptor stimulation has been reported to activate CAMKII *via* a PKA-independent activation of the L-type Ca^{2+} channel in adult rat cardiac myocytes (Zhu *et al* 2003; Wang *et al* 2004). In the perfused heart only β_1 receptor stimulation and not β_2 stimulation caused phosphorylation of PLB at Thr17 (CAMKII site), suggesting CAMKII is not a downstream target of the β_2 receptor (Bartel *et al* 2003). CAMKII has also been shown to be activated by α_1 stimulation in cardiac myocytes (O-Uchi *et al* 2005; Wang *et al* 2001a). In adult rat cardiac myocytes, the phenylephrine-induced activation of CAMKII was inhibited by a PKC inhibitor (O-Uchi *et al* 2005). The role of PKC in the activation of CAMKII is unknown. In the same study, CAMKII was also shown to translocate from the plasmalemma to transverse tubules (O-Uchi *et al* 2005). The authors suggested that like PKC, the translocation may be important for the regulation of CAMKII activity .

1.5.5 Phosphoinositide-3-kinase (PI3K)

PI3Ks are lipid kinases that phosphorylate the inositol ring of phosphatidylinositol (PtdIns) and various phosphatidylinositol phosphates at the 3 position. PI3Ks are grouped into three classes; classes I, II, and III. *In vivo*, class I PI3Ks predominantly phosphorylate PtdIns(4,5)P_2 to generate PtdIns(3,4,5)P_3, whereas class II and III mainly phosphorylate PtdIns to generate PtdIns(3)P (Oudit *et al* 2004). The class I PI3Ks are heterodimeric enzymes consisting of catalytic and regulatory adaptor subunits, and they are further divided into class IA and IB. There are three isoforms for class IA catalytic subunits; p110α, p110β, and p110δ, and three isoforms for the adaptor subunit; p85α, p85β, and p55γ. The class IB PI3K (PI3Kγ) is composed of the catalytic subunit p110γ tightly bound to the regulatory subunit p101. The adaptor subunits for the class IA PI3Ks contain a SH2 domain as well as a p110 binding domain, and they recruit the catalytic subunit to the plasma membrane upon tyrosine phosphorylation following, for example, receptor tyrosine kinase activation by insulin. In contrast, the regulatory subunit of PI3Kγ lacks the SH2 domain. Instead,

both the catalytic and regulatory subunits of PI3Kγ contain the binding sites for $G_{\beta\gamma}$ (Wymann *et al* 2003). PI3Kγ is recruited and activated by $G_{\beta\gamma}$ following activation of GPCRs (Stephens *et al* 1997; Alloatti *et al* 2004; Naga Prasad *et al* 2000). Both class IA PI3Ks and PI3Kγ catalytic subunits also contain the C2 domain involved in phospholipid binding, the PIK domain involved in protein-protein interaction, and the Ras-binding domain. Ras-GTP binding enhances the catalytic activity of both class IA PI3K and PI3Kγ (Wymann *et al* 2003).

The class IA PI3Kα and PI3Kβ enzymes are expressed in cardiac myocytes (Crackower 2002). The physiological importance of PI3Kγ in cardiac myocytes is questioned by some investigators because it is only weakly expressed (Alloati *et al* 2004). However deletion of PI3Kγ causes significant differences in the cardiac phenotype including altered contractile function in response to catecholamines and the loss of downstream effects of some GPCRs, suggesting the physiological importance of this protein (Alloatti *et al* 2004). The signalling and physiological function of class IA PI3Ks and PI3Kγ appear to be distinct. While class IA PI3Ks seem to be involved in adaptive hypertrophy without cardiac dysfunction, PI3Kγ is associated with pathological hypertrophy (Shioi *et al* 2000; Naga Prassad *et al* 2003). Moreover, only PI3Kγ appears to be involved in the regulation of contraction (Alloatti *et al* 2004).

Several studies have reported the activation of PI3K after β_2 receptor stimulation in neonatal rat cardiac myocytes or in adult rat or mouse cardiac myocytes (Chesley *et al* 2000; Jo *et al* 2002; Zhu *et al* 2001). The stimulation of the β_1 receptor has also been reported to activate PI3K in adult rat cardiac myocytes (Leblais *et al* 2004). However, the isoforms of PI3K activated in these studies were not determined, and the pathways by which β receptors activate PI3K are not completely understood. Figure 1.1 summarises the putative pathways by which the β receptor activates PI3K in cardiac myocytes. For the β_2 receptor, the observations that the activation of PI3K or its downstream effects were inhibited by PTX or βARK-ct peptide (an inhibitor of $G_{\beta\gamma}$ signalling) suggest the activation of PI3Kγ through G_i and $G_{\beta\gamma}$ (Chesley *et al* 2000; Jo *et al* 2002). In contrast, the downstream effects of PI3K activation by the β_1 receptor were unaffected by PTX, although they were inhibited by βARK-ct (Leblais *et al* 2004). The lack of involvement of G_i is consistent with the exclusive coupling

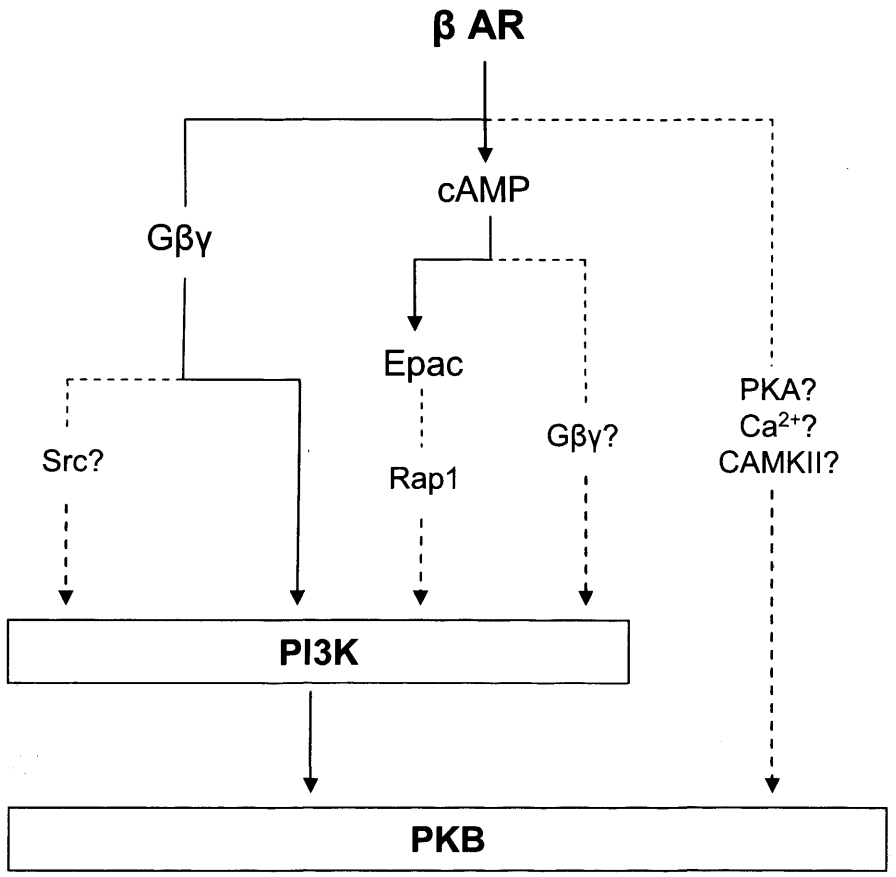


Figure 1.1 Putative signalling pathways linking the β adrenergic receptor to PI3K and PKB

Dotted lines indicate pathways which have been suggested in cardiac mycocytes but have not been completely elucidated, or pathways which have been observed in non-cardiac myocytes but yet to be confirmed in cardiac myocytes.

of the β_1 receptor with Gs. It is possible that the β_1-mediated activation of PI3K is due to activation of PI3Kγ by $G_{\beta\gamma}$ derived from G_s. However, the involvement of $G_{\beta\gamma}$ does not necessarily prove the selective activation of PI3Kγ. As well as being the direct activator of PI3Kγ, $G_{\beta\gamma}$ has also been reported to be upstream of non-receptor tyrosine kinases such as Src family tyrosine kinases, and an increase in tyrosine kinase activity after β receptor stimulation has been demonstrated in cardiac myocytes (Zou *et al* 1999). Thus, the possibility of activation of the class IA PI3Ks by β receptors through a $G_{\beta\gamma}$-mediated increase in tyrosine phosphorylation cannot be ruled out (Morisco *et al* 2000).

In adult cardiac myocytes, forskolin has also been shown to activate PI3K (Leblais *et al* 2004), suggesting cAMP signalling may also contribute to the PI3K activation by β receptors. The activation of PI3K by forskolin has been indirectly shown to be inhibited by βARK-ct, suggesting the involvement of $G_{\beta\gamma}$. How cAMP signalling affects $G_{\beta\gamma}$ signalling and activates PI3K is not clear, but it may be related to the effect of PKA to phosphorylate the β_2 receptor and increase the coupling of the β_2 receptor to G_i (Section 1.5.9). It has been shown in HEK293 cells that the expression of Epac activates PI3K *via* Rap1 (Mei *et al* 2002). Whether PI3K is activated by cAMP through Epac in cardiac myocytes is unknown.

Till *et al* have reported the possibility of contraction-dependent activation of PI3K in cardiac myocytes (Till *et al* 2000). These investigators observed that electrical contraction of adult rat cardiac myocytes increased the PI3K activity present in the cell lysate (Till *et al* 2000). This activation of PI3K was not associated with the tyrosine phosphorylation of the insulin receptor and insulin receptor substrates (IRS) 1/2 or the recruitment of PI3K to IRS1/2 (Till *et al* 2000). However, they observed increased association of p85α with unidentified protein which is tyrosine-phosphorylated following the myocyte contraction (Till *et al* 2000). This contraction-mediated activation of PI3K may be relevant to the adrenergic regulation of PI3K activity in the beating heart/myocytes or in the *in vivo* heart.

Compared to the β receptor, the information regarding activation of PI3K by α_1 receptor stimulation in the heart is limited. Doenst and Taegtmeyer reported that the effects of the α_1 receptor and insulin to increase glucose uptake in the perfused heart

were additive but both effects were inhibited by wortmannin (Doenst and Taegtmeyer 1999). This suggests that the cardiac α_1 receptor activates a PI3K isoform, possibly PI3Kγ, which is different from those activated by insulin.

1.5.6 Protein kinase B (PKB) / Akt

The activation of PKB by PI3K involves the recruitment of PKB to the plasma membrane through the interaction of PtdIns(3,4,5)P_3 with the PH domain, and phosphorylation by the phosphoinositide-dependent kinase 1 (PDK1), which is also recruited to the membrane by the PH domain. PDK1 phosphorylates PKB at Thr308 in the catalytic domain (Mora *et al* 2004). PtdIns (3,4,5)P_3 is thought to promote the phosphorylation of PKB by PDK1 by causing a conformational change of PKB which exposes Thr308 and by bringing constitutively active PDK1 to the proximity of PKB (Alessi *et al* 1997 and 1998; Stokoe *et al* 1997; Anderson *et al* 1998). PKB is additionally phosphorylated at Ser473 in the C-terminal hydrophobic region ('the PDK2 site'). The identity of the kinase(s) that phosphorylate(s) Ser478 is the subject of intense investigation (Dong and Liu 2005). Several candidate kinases, including mTOR/rictor complex, have been reported (Dong and Liu 2005). The Thr308 phosphorylation is essential for PKB activity. The role of Ser473 is less clear but it may stabilise the active configuration and/or it may assist the phosphorylation of Thr308 by PDK1 (Dong and Liu 2005). However, Thr308 phosphorylation has been seen in the absence of Ser473 (Morisco *et al* 2005).

In neonatal and adult rat cardiac myocytes, isoproterenol or β_2 receptor stimulation has been shown to increase PKB activity (as measured by GSK3α phosphorylation) and/or phosphorylation of Ser478 (Morisco *et al* 2000; Zhu *et al* 2001). It has been reported that phenylephrine does not affect Ser478 phosphorylation of PKB in adult rat cardiac myocytes, suggesting PKB may not be downstream of the α_1 receptor (Wang *et al* 2001b). This apparently contradicts the activation of PI3K by the α_1 receptor (Section 1.5.5). However, the lack of PKB phosphorylation under conditions that increase PI3K activity has been reported by several other studies (Till *et al* 2000; Zou *et al* 2002 and 2003) and it may be due to compartmentation of PI3K signalling. The activation of PKB by the β receptor is sensitive to inhibitors of PI3K (Morisco *et*

al 2000; Zhu *et al* 2001). However, several observations suggest that PKB may also be activated by PI3K-independent pathways (Figure 1.1). It has been shown in 293 cells that cAMP-raising agents activate PKB by a PKA-dependent but PI3K-independent pathway (Fillippa *et al* 1999). The activation of PKB by cAMP-raising agents was associated with increased phosphorylation of Thr308 but it was seen in the absence of Ser473 phosphorylation (Fillippa *et al* 1999). This is in contrast to the activation of PKB by insulin, which required phosphorylation of both Thr308 and Ser473 (Fillippa *et al* 1999). It appears that the increase in Thr308 phosphorylation by cAMP-raising treatments is not due to direct phosphorylation of PKB by PKA (Fillippa *et al* 1999). The possibility that PKB is also activated by the PKA pathway in cardiac myocytes is supported by the recent study by Morisco *et al* (Morisco *et al* 2005). These investigators showed that isoproterenol caused phosphorylation of PKB at Thr308 and Ser473 with different time-courses; the phosphorylation of Thr308 could be detected within 1 minute of stimulation and it returned to the basal level after 60 minutes, whereas the phosphorylation of Ser473 increased after 10 minutes and was sustained for 2 hours (Morisco *et al* 2005). They showed that the early phosphorylation of Thr308 (10 minutes), but not the later phosphorylation of Ser473 (60 minutes), was insensitive to wortmannin (Morisco *et al* 2005). The phosphorylation of Thr308 was blocked by H89, nifidepine, or KN93, suggesting the possibility that PKA regulates PKB phosphorylation through activation of the L-type Ca^{2+} channel and CAMKII (Morisco *et al* 2005). How Ca^{2+}/CAMKII might be linked to PKB is not clear.

1.5.7 Protein kinase C (PKC)

There are at least ten different isoforms of PKC, which are grouped into conventional (cPKC) (α, βI, βII, γ), novel (nPKC) (δ, ε, η, θ), and atypical (aPKC) (ζ, λ) PKCs. All PKC isoforms share conserved C-terminal catalytic domains termed C3 and C4 but display differences in the N-terminal regulatory domain. The regulatory domain of the cPKCs contains two tandem repeats of a cysteine-rich zinc finger-like motif termed C1 which binds DAG, and the region termed C2 which binds membrane phospholipids in a Ca^{2+} dependent manner. Both Ca^{2+} and DAG are required for the activation of the cPKC isoforms. DAG increases the affinity of cPKCs for Ca^{2+}

(Nishizuka 1984). The nPKCs also contain the C1 region but they lack the C2 region. These isoforms require DAG for activation but are insensitive to Ca^{2+}. Both cPKCs and nPKCs are also activated by phorbol esters, which are DAG analogues. The aPKCs, which lack the C2 region and contain only one cysteine-rich zinc finger-like motif, are insensitive to DAG or Ca^{2+}. All PKC isoforms, however, require phosphatidylserine (PS) for activity. The activation of PKC isoforms is associated with translocation from the cytosolic to a particulate subcellular fraction. For the cPKCs and nPKCs, the translocation to the membrane and the interaction with DAG and PS provides the energy to release the inhibitory pseudosubstrate from the active site. Differential centrifugation followed by detection with PKC isoform-specific antibodies has been most commonly used to show the activation of particular PKC isoforms. Anchoring proteins called Receptors for Activated C-kinases (RACKs) target activated PKC isoforms to a specific subcellular location (Mochly-Rosen and Gordon 1998). In neonatal cardiac myocytes RACK1 selectively targets activated PKCβII to the perinuclear structure (Ron *et al* 1995). RACKII (beta'-COP) selectively targets activated PKCε to the Golgi membrane in cardiac myocytes (Csukai *et al* 1997). It has been suggested some of the inactive PKC isoforms are also selectively targeted to different subcellular sites by Receptor for Inactive C-kinases (RICKs) (Mochly-Rosen and Gordon 1998).

DAG, which is required for cPKCs and nPKCs activation, initially comes from the hydrolysis of PtdIns(4,5)P_2 by PLCβ upon G_q-coupled receptor activation (Nishizuka 1995). However, DAG derived from PLCβ is quickly degraded by the actions of DAG kinase and DAG lipase (Nishizuka 1995). The sustained increase in DAG, which is required for some of the long term effects of G_q signalling such as proliferation, is thought to be due to the hydrolysis of phosphatidylcholine (PC) (Nishizuka 1995). This is because the fatty acid composition of DAG, which is sustained, corresponds with that of PC, and DAG derived from PC is more slowly degraded than DAG derived from PtdIns(4,5)P_2 (Nishizuka 1995). DAG is produced from PC by the action of phospholipase D (PLD) which hydrolyses PC to generate phosphatidic acid (PA), which is dephosphorylated by phosphatidate phosphohydrolase to DAG. PA is also known to be an activator of aPKCs (Farese *et al* 2002). PLD has been shown to be activated by α_1 receptor stimulation in the rat heart (Weismuller *et al* 2004).

In addition to DAG and Ca^{2+}, the activity of PKC is also regulated by cis-unsaturated fatty acids (Nishizuka 1995). These fatty acids are incapable of activating cPKCs on their own but they increase the affinity of cPKCs for Ca^{2+} synergistically with DAG, causing the activation of the PKCs at basal Ca^{2+} level (Shinomura *et al* 1991; Yoshida *et al* 1992). The cis-unsaturated fatty acids including arachidonic acid activate some nPKCs (ε and δ) either in a DAG-dependent manner or independently of DAG (Koide *et al* 1992; Ogita *et al* 1992). PKCζ has also been reported to be activated by arachidonic acid (Kochs *et al* 1993). Phospholipase A_2 (PLA_2) appears to play a role in PKC signalling by hydrolysing the sn-2-fatty acyl ester bonds thereby releasing free fatty acids and lysophospholipids. Lysophosphatidylcholine, produced by the hydrolysis of PC by PLA_2, is also known to enhance the DAG-dependent activation of cPKC (Sasaki *et al* 1993). There are several PLA_2 types which display differences in their preference for the sn-2-fatty acids, subcellular localisation, and Ca^{2+} requirement (Pavoine and Defer 2005). The cytosolic PLA_2, which selectively releases arachidonic acid, has been shown to be activated by α_1 or β_2 receptors (Pavoine *et al* 2003; Ait-Mamar *et al* 2005; Debetto *et al* 1999).

Some members of nPKCs (δ, ε, and η) and aPKCs are also known to be activated by PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Farese *et al* 2002; Rameh and Cantley 1999; Toker *et al* 1994; Nakanishi *et al* 1993). The activation by PtdIns(3,4,5)P_3 is partly mediated by the phosphorylation of nPKCs and aPKCs by PDK1 (below).

All PKC isoforms are phosphorylated at the threonine residue in the activation loop and at one (aPKCs) or two (cPKCs and nPKCs) residues at the C-terminus (Malhotra *et al* 2001b). The activation loop threonine of all three groups of PKCs is phosphorylated by PDK1 (Dutil *et al* 1998, Le Good *et al* 1998). For cPKCs, the phosphorylation of the activation loop by PDK1 and subsequent stimulation of autophosphorylation at the two C-terminal residues are required for the maturation of newly synthesised cPKCs (Dutil *et al* 1998). cPKCs phosphorylated at the C-terminal are still inactive but are now capable of being activated by Ca^{2+} and DAG (Dutil *et al* 1998). Once cPKCs are in the mature form, the activation loop can be dephosphorylated without affecting the activation of cPKCs by Ca^{2+} and DAG (Dutil *et al* 1998). The re-phosphorylation of the activation loop of the mature cPKCs by PDK1 does not change the basal activity or DAG- and Ca^{2+}-induced activity (Dutil *et*

al 1998). In contrast to cPKCs, PDK1 phosphorylation of the activation loop increases the activity of aPKCs in the presence of PS or the activity of nPKCs in the presence of PS and DAG (Le Good *et al* 1998). This activation of aPKCs and nPKCs is stimulated in the presence of PtdIns(3,4,5)P_3 (Le Good *et al* 1998). However, phosphorylation of aPKCs by PDK1 is not an essential mechanism for the activation of aPKCs because PA appears to activate aPKCs without this phosphorylation (Farese *et al* 2002).

The nature of the PKC isoforms expressed in cardiac myocytes is controversial. Adult rat cardiac myocytes have been reported to express α, β1, β2, δ, ε, and ζ (Malhotra *et al* 2001a). However studies by another group found that adult cardiac myocytes express PKCε and ζ but they do not express α, β1, γ, or δ (Bogoyevitch *et al* 1993). In cultured neonatal rat myocytes, PKCα, δ, ε, and ζ were detected but PKCβ was not detected (Puceat *et al* 1994). A number of studies done with transgenic mice and isolated cardiac myocytes have shown that α_1 receptor/G_q stimulation selectively increases the translocation of PKCε to the membrane without affecting the translocation of PKCα and PKCζ (D'Angelo *et al* 1997; Puceat *et al* 1994; Clerk *et al* 1994; Wang *et al* 2003; Deng *et al* 1998). Whether PKCδ is activated by α_1 receptor stimulation is controversial. The translocation of PKCδ upon α_1 stimulation has been observed by some groups (Puceat *et al* 1994; Wang *et al* 2003; Deng *et al* 1998), but not by others (Clerk *et al* 1994). There have been studies suggesting that β receptor stimulation can also activate PKC. The signalling linking the β receptor to PKC is unknown but it appears to involve Ca^{2+}. In the perfused rat heart, isoproterenol pre-treatment (10 min) provided ischemic preconditioning which was inhibited by a L-type Ca^{2+} channel blocker or by PKC inhibitors (Miyawaki and Ashraf 1997). In adult rat cardiac myocytes isoproterenol (1h) increased translocation of PKCε to the membrane without affecting PKCα and δ (Shizukuda and Buttrick 2001).

1.5.8 Protein kinase D (PKD)

PKD is a family of serine/threonine kinases that includes PKD, PKD2 and PKD3 (Wang 2006). Although PKD and PKD3 are also known as PKCμ and PKCν,

respectively, PKDs show different substrate specificities from PKCs and are more closely related to CAMKs (Wang 2006). PKD contains a PH domain and the N-terminal C1 domain (C1a/C1b) which binds DAG and phorbol esters. Like conventional and novel PKCs, PKD is activated by DAG or phorbol ester (van Lint 1995). PKDs are also activated by phosphorylation by PKC (particularly the novel PKCs) which relieves the autoinhibitory effect of the PH domain (Wang 2006). PKD is expressed in rat ventricular myocytes and is activated by GPCR (Haworth *et al* 2000) but the cardiac function of PKDs is still not well-understood. PKD has been suggested to mediate the effect of PKC to phosphorylate class II histone deacetylase (Vega *et al* 2004). Cardiac troponin I and myosin-binding protein C have been identified as possible PKD targets (Haworth *et al* 2004), indicating a possible role of PKD in the regulation of contraction.

1.5.9 Mitogen-activated protein kinases (MAPKs)

MAPKs are classified into 5 groups (Michel *et al* 2001);

1. The extracellular signal-regulated kinase (ERK) 1 and 2
2. p38 α, β, γ, and δ
3. The c-Jun N-terminal kinase (JNK) 1, 2, and 3
4. ERK 5
5. ERK 3 and 4

The regulation and function of the first three groups are most extensively studied whereas the functional information for the last two groups is relatively scarce. The heart predominantly expresses ERK1/2, p38α/β, and JNK1/2 (Magne *et al* 2001; Lemke *et al* 2001; Stein *et al* 1997; Clerk *et al* 1998). MAPKs are activated by phosphorylation of threonine and tyrosine in the Thr-X-Tyr motif in the activation loop by one or more of the upstream dual specificity kinases, MAPK kinases (MKKs) which selectively activate each group of MAPKs. Thus, MEK1 and MEK2 activate ERKs, MKK3 and MKK6 activate p38s, and MEK4 and MEK7 activate JNKs. MAPKs are inactivated by specific MAPK dual phosphatases. The activity of MKKs is under the control of the upstream ser/thr kinase, MAPK kinase kinases

(MKKKs). The Raf MKKK family (Raf1, A-Raf, and B-Raf) and MEKK1 are upstream components of the ERK MAPK cascade. MKKKs for p38 include JAK1 and PAK, and those for JNK include MEKK4, MUK, and MST. MKKKs often activate more than one group of MAPKs by activating several MKKs. Thus, MEKK2 and MEKK3 can activate both ERK and JNK *via* their respective MKKs. Similarly ASK1, SPRK, TAK1 can activate both p38 and JNK.

In cardiac myocytes, both α_1 and β receptors have been reported to activate all three of ERK, p38, and JNK (Markou and Lazou 2002; Yamazaki *et al* 1997; Communal *et al* 2000; Wang and Proud 2002; Maruyama *et al* 2002). However, there is considerable controversy over the activation of individual MAPKs (ERK, p38, or JNK) by different adrenergic receptor types and subtypes, and the relative importance of each MAPK on the development of hypertrophy and apoptosis (Michel *et al* 2001). This is at least partly due to diversity of the experimental systems employed (transgenic animals, freshly isolated/cultured myocytes, perfused hearts) and the age (adult vs. neonatal myocytes) and species (e.g. rat vs. mouse) specificity of the adrenergic signalling.

The adrenergic receptors activate MAPKs through multiple pathways which are not completely understood. Figure 1.2 summarises some of the pathways that have been suggested to be involved in MAPK activation. Many studies have identified PKC as a key signalling component mediating the activation of ERK and possibly other MAPKs by the α_1 receptor (Sugden and Clerk 1998b; Zou *et al* 1999; Yamazaki *et al* 1997; Bogoyevitch *et al* 1996; Wang *et al* 2003). The activation of ERK by PKC appears to involve Raf (Sugden and Clerk 1997). In addition, the activation of MAPK by the α_1 receptor has been suggested to involve the small G proteins Ras and Rho (Thorburn 1994; Sugden and Clerk 2000). Rho has been shown to be downstream of G_{12} and G_{13}, and it seems to be involved in the activation of JNK by the α_1 receptor (Maruyama *et al* 2002). G_{12} and G_{13} may activate Rho by stimulating the GEF activity of AKAP-Lbc (Kurose 2003; Diviani *et al* 2001) and p115RhoGEF (Hart *et al* 1998), respectively.

In contrast to the α_1 receptor, the activation of ERK by the β receptor is insensitive to PKC inhibition (Zou *et al* 1999; Bogoyevitch *et al* 1996; Yamazaki *et al* 1997).

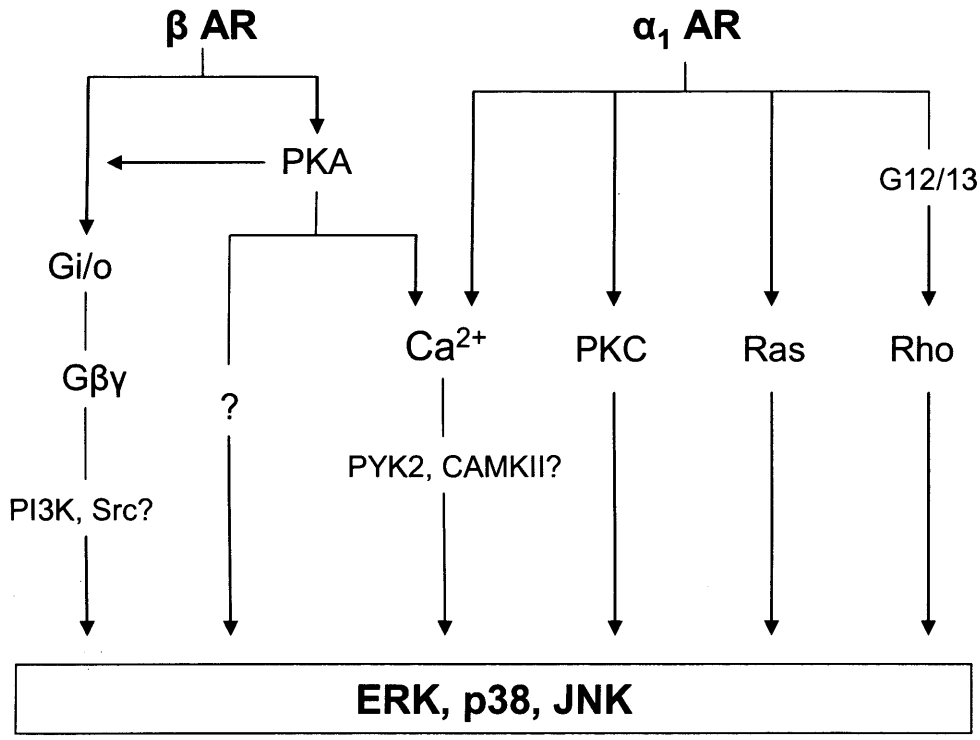


Figure 1.2 Signalling pathways linking cardiac adrenergic receptors to MAPKs

Instead, the activation of MAPK by the β receptor appears to involve PKA, $G_{i/o}$, and $G_{\beta\gamma}$ signalling (Zou *et al* 1999; Yamazaki *et al* 1997; Zheng *et al* 2000; Chesley *et al* 2000; Communal *et al* 2000), although the pathways linking these to MAPK are not well-understood. $G_{\beta\gamma}$ has been suggested to activate MAPK *via* PI3Kγ (Lopez-Ilasaca *et al* 1997). It has also been reported that G_i-derived $G_{\beta\gamma}$ activates ERK *via* the Src-Ras-Raf1 pathway, and phosphorylation of the β_2 receptor by PKA stimulates this pathway by increasing the coupling of the receptor with G_i (Zou *et al* 1999; Daaka *et al* 1997). However, how $G_{\beta\gamma}$ activates the Src pathway is not clear. Possibly, it could involve the activation of βARK and the recruitment of β-arrestin, which has been postulated to activate MAPK by binding Src as well as the components of ERK and JNK MAPK cascades (Rockman *et al* 2002). The activation of p38 by the β_2 receptor has been shown to be dependent on PKA but independent of $G_i/G_{\beta\gamma}$ (Zheng *et al* 2000), suggesting PKA also activates MAPK *via* mechanisms other than the stimulation of the G_i pathway. One likely pathway is the increase in intracellular Ca^{2+} through activation of the L-type Ca^{2+} channel. The activation of ERK by isoproterenol or phenylephrine has been reported to be inhibited by Ca^{2+} chelation or nifedipine, suggesting Ca^{2+} is a common mechanism mediating the activation of MAPK by both α_1 and β receptors (Yamazaki *et al* 1997; Bogoyevitch *et al* 1996). The mechanisms by which Ca^{2+} activates MAPK are not clear. The possible downstream pathways include the activation of tyrosine kinase PYK2 (Sugden and Clerk 1997) or CAMKII, which appears to be upstream of the apoptosis signal-regulated kinase 1 (ASK1) (Kashiwase *et al* 2005).

1.6 AMP-activated protein kinase (AMPK)

1.6.1 Introduction

The AMP-activated protein kinase is a broadly expressed serine/threonine kinase consisting of catalytic α subunit and regulatory β and γ subunits. AMPK and its orthologues are present in all eukaryotes. The earlier studies with purified AMPK from the rat liver (much of the earlier *in vitro* studies were done with AMPK from this tissue) showed AMPK was activated by AMP and by phosphorylation of Thr172 at the catalytic loop of the α subunit by upstream kinases, collectively called the

AMPK kinases (AMPKKs), which cause a dramatic increase in the AMPK activity (Hawley *et al* 1996). AMP activates AMPK by several mechanisms; 1. It binds to AMPK and allosterically activates AMPK (Sullivan *et al* 1994). 2. It enhances the phosphorylation of Thr172 by AMPKKs (Hawley *et al* 1995). 3. It protects AMPK against the dephosphorylation of Thr172 by PP2C and PP2A, the phosphatases identified to dephosphorylate Thr172 *in vitro* (Davis *et al* 1995). 4. It allosterically activates AMPKK (Hawley *et al* 1995). The last effect was observed with a partially purified liver AMPKK but it is questioned following the recent discoveries that LKB1 and CAMKKβ phosphorylate Thr172 and act as AMPKKs (Section 1.6.3). Both LKB1, which is the main AMPKK in the liver, and CAMKKβ are not directly activated by AMP (Section 1.6.3). All four effects of AMP have been shown to be antagonised by ATP (Carling *et al* 1989; Davis *et al* 1995; Hawley *et al* 1996). Thus, the activity of AMPK *in vivo* is likely to be regulated by changes in the AMP/ATP ratio rather than AMP *per se*. A number of conditions that increase the cellular AMP/ATP ratio (e.g. skeletal muscle contraction and H_2O_2) have been demonstrated to increase Thr172 phosphorylation and the AMPK activity in isolated or *in vivo* tissues/cells. In the heart, AMPK has been shown to be activated by ischemia and by metabolic inhibitors which impede ATP production (Kudo *et al* 1995; Pelletier *et al* 2005). AMPK is also activated in many cell types including cardiac myocytes (Chabowski *et al* 2006) by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (Corton *et al* 1995), which is converted in the cell to ZMP, an AMP analogue which has also been shown to activate AMPK *in vitro* (Sullivan *et al* 1994). AICAR is the most commonly used experimental tool for activating AMPK, and many downstream effects of AMPK have been studied using this compound.

In the heart AMPK is emerging as a key regulator of many metabolic pathways. AMPK has been shown to phosphorylate and inactivate cardiac ACC2 (Dyck *et al* 1999). AMPK has also been shown by some investigators to phosphorylate and activate MCD in skeletal muscle (Saha *et al* 2000) but this effect has not been confirmed by others (Habinowski *et al* 2001). The activation of AMPK during ischemia/reperfusion is associated with phosphorylation of ACC, a decrease in malonyl CoA, and an increased rate of fatty acid oxidation (Kudo *et al* 1995 and 1996). AMPK activation is also associated with increased lipoprotein lipase activity (An *et al* 2005) and translocation of the fatty acid translocase CD36 to the plasma

membrane (Luiken *et al* 2003). Recently, prolonged activation of AMPK has been shown to increase the expression of fatty acid transporters FABPpm and FAT/CD36 in cardiac myocytes and perfused heart (Chabouski *et al* 2006). In addition to stimulating fatty acid utilisation, AMPK also regulates glucose metabolism. Several studies have shown that the activation of AMPK increases the translocation of Glut4 to the plasma membrane (Russell *et al* 2004; Li *et al* 2005). AMPK has also been shown to phosphorylate and activate PFK2 *in vitro* and this is implicated in the stimulation of glycolysis during ischemia (Marsin *et al* 2000). Results from skeletal muscle suggest that AMPK may also regulate cardiac glycogen metabolism by inhibiting glycogen synthase (Wojtaszewski *et al* 2002). AMPK possibly stimulates glycogen phosphorylase (by phosphorylating phosphorylase kinase) but this effect is controversial (Carling and Hardie 1989; Young *et al* 1996; Beyer *et al* 2000). These effects of AMPK to stimulate energy producing pathways, together with the activation of AMPK by an increase in the AMP/ATP ratio, have led to the notion that the main physiological function of this enzyme is to protect the cells against the loss of ATP during metabolically stressful conditions. For this reason AMPK is described as the energy or stress sensor (Hardie *et al* 2006). This role of AMPK has been supported by the recent study with hearts from transgenic mice lacking α2 AMPK (the major isoform), which showed a much higher increase in AMP/ATP during ischemia than wild type hearts (Zarrinpashneh *et al* 2006).

In addition to stimulating energy producing pathways, the maintenance of cellular ATP by AMPK is also likely to involve inhibition of energy consuming pathways. Liver ACC and HMG CoA reductase, which are involved in the biosynthesis of fatty acids and cholesterol, respectively, are inactivated by AMPK (Carling *et al* 1987). In the heart, which is not lipogenic, one relevant biosynthetic pathway which is inhibited by AMPK activation is protein synthesis (Chan and Dyck 2005). The activation of AMPK in cardiac myocytes causes inhibition of protein synthesis by phosphorylation and activation of the eukaryotic elongation factor 2 (eEF2) kinase, which phosphorylates and inhibits eEF2 (Horman *et al* 2003; Browne *et al* 2004; Macleod *et al* 2002; Chan *et al* 2004; Browne and Proud 2004). Studies in cardiac myocytes and other cell types have suggested that AMPK also regulates protein synthesis by inhibiting the mTOR-p70 S6 kinase pathway (Bolster *et al* 2002; Kimura *et al* 2003). AMPK may inhibit this pathway either by direct inhibitory

phosphorylation of mTOR (Cheng *et al* 2004), or by phosphorylation and activation of TSC2, which is a negative regulator of mTOR (Inoki *et al* 2003).

Recently, there have been an increasing number of studies reporting AMP/ATP-independent modulation of AMPK activity and Thr172 phosphorylation state. AMPK has been demonstrated to be activated by hyperosmotic stress (Section 1.6.5.d) and metformin (Section 1.6.5.f) *via* AMP/ATP-independent mechanisms. It has also been reported that AMPK is inactivated by glucose in skeletal muscle (Section 1.6.5.l) and is activated by long chain fatty acids in the heart and skeletal muscle (Section 1.6.5.m) through an AMP/ATP-independent change in the Thr172 phosphorylation state. These findings suggest that, in addition to protecting cells from ATP depletion, AMPK may have other roles. For example, AMPK may act as a fuel sensor which regulates fuel metabolism in response to the change in the type of fuel supplied. Moreover, recent studies have also shown that the activity and the Thr172 phosphorylation state of AMPK can be modulated by a number of hormonal or other extracellular signals through the stimulation of cell surface receptors. These include insulin (Section 1.6.5.e), adiponectin (Section 1.6.5.i), leptin (Section 1.6.5.i), ghrelin (Section 1.6.5.j), resistin (Section 1.6.5.i), extracellular nucleotides (Section 1.6.5.h), fibronectin (Han *et al* 2006), angiotensin II (Nagata *et al* 2004), histamine (Section 1.6.5.h), thrombin (Section 1.6.5.h), interleukin-6 (Kelly *et al* 2004) and adrenergic agonists (Section 1.6.5.p). These observations suggest that AMPK may additionally mediate some of the biological effects of these signals.

1.6.2 AMPK structure

AMPK purified from rat liver exists as a heterotrimer of α, β, and γ subunits (Davies *et al* 1994). *In vitro* expression of the three subunits showed that the formation of the ternary complex is not dependent on phosphorylation or the catalytic activity of the α subunit (Woods *et al* 1996; Dyck *et al* 1996). The β subunit interacts with both α and γ subunits, but the α subunit does not interact (Woods *et al* 1996) or interacts very weakly with the γ subunit (Dyck *et al* 1996). Transfection of the AMPK subunits in COS-7 cells has shown that co-expression of either the β or γ subunit with the α subunit has a small stimulatory effect on AMPK activity but the full activity requires

the expression of all three subunits (Dyck *et al* 1996). This may be partly due to the stabilisation of the α subunit by the β and γ subunits (Dyck *et al* 1996). The homologues of all three subunits are found in S.*cerevisiae*. The AMPK α, β, and γ subunits are genetically and functionally related to the yeast Snf1p, Sip1p/Sip2p/Gal83p, and Snf4p, respectively (Stapleton *et al* 1994; Yang *et al* 1994; Woods *et al* 1996; Carling *et al* 1994; Woods *et al* 1994).

The catalytic α subunit (Carling *et al* 1989) transfers γ phosphate of ATP to serine or threonine residues in the consensus sequence (Φ(X,β)XXS/T*XXXΦ, where Φ and β indicate hydrophobic and basic residues, respectively; X indicates any amino acid; the order of the amino acids within the parenthesis is not important) (Weekes *et al* 1993; Dale *et al* 1994; Salt *et al* 1998). However, a structural modelling of the interaction between the catalytic domain of AMPK and the region around Ser79 (AMPK phosphorylation site) of ACC1 has suggested that residues over a much longer region than the above recognition motif are important for the interaction (Scott *et al* 2002). There are two isoforms of the α subunit; α1 (548 residues) encoded by the gene on chromosome 5p11 (Stapleton *et al* 1997) and α2 (552 residues) encoded by the gene on chromosome 1p31 (Beri *et al* 1994) (Stapleton *et al* 1996). Both isoforms migrate on SDS-PAGE at approximately 63kDa and are activated by AMP and by the phosphorylation of Thr172 by AMPKK (Stapleton *et al* 1996). They show high sequence similarity at the N-terminal catalytic domain but less similarity at the C-terminal region (Stapleton *et al* 1996). Truncation studies have shown that the C-terminal region (313-473) of the α subunit is required for association with the β and γ subunits (Figure 1.3) (Crute *et al* 1998; Iseli *et al* 2005). A truncated α1 lacking the C-terminal region (α1 (312)) is constitutively active and the presence of an autoinhibitory domain has been suggested in the region between residues 312-392 (Crute *et al* 1998). The α1 mRNA and protein are generally expressed but α2 mRNA and protein are most abundant in heart, skeletal muscle and liver (Stapleton *et al* 1996). In terms of the activity, α2 AMPK activity is predominant in the heart and skeletal muscle, while α1 AMPK activity is predominant in lung, kidney, testis, brain and pancreas (Cheung *et al* 2000). Both α1 and α2 AMPK contribute equally in the liver (Cheung *et al* 2000). In the heart, α1 AMPK contributes to about 30% of the total AMPK activity (Cheung *et al* 2000).

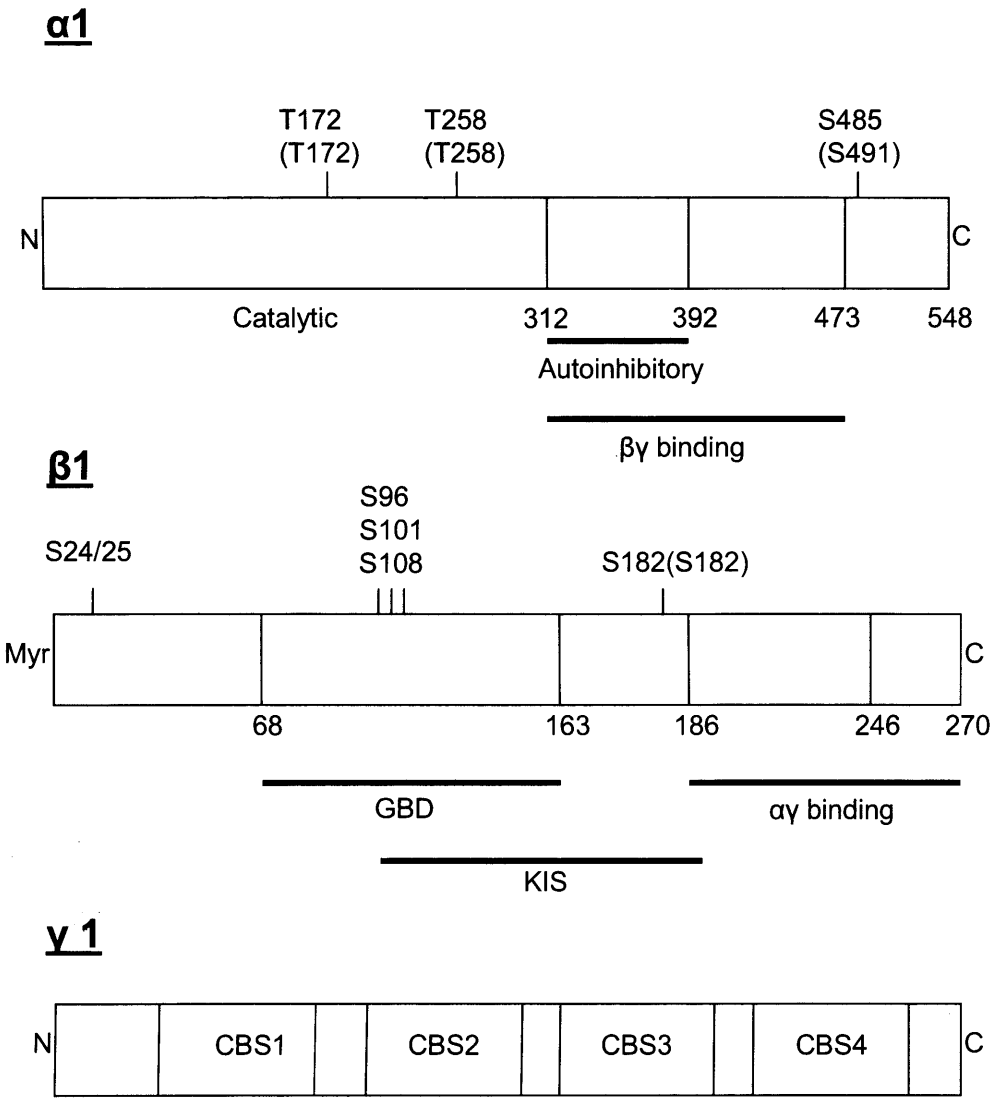


Figure 1.3 AMPK subunit domain structures and phosphorylation sites

The domain structure and the phosphorylation sites for the α1, β1, and γ1 subunits are shown. The phosphorylation sites in parentheses represent the corresponding phosphorylation sites in the α2 and β2 subunits.

The α1 and α2 isoforms display a number of differences. The α2 activity is stimulated by AMP to a greater extent than α1 *in vitro* (Salt *et al* 1998). This effect was seen for both the allosteric effect of AMP and for the stimulation of the Thr172 phosphorylation by an upstream kinase. This difference in the sensitivity to AMP may explain the greater activation of α2 AMPK than α1 AMPK during cardiac ischemia (Li *et al* 2006) and the isoform-specific AMPK activation during skeletal muscle contraction. Low or moderate skeletal muscle contraction has been generally found to selectively activate α2 AMPK while α1 activation is only seen during high intensity contraction (Vavvas *et al* 1997; Stephens *et al* 2002; Wojtaszewski *et al* 2000; Chen *et al* 2000), although a recent study by Toyoda *et al* has shown a selective activation of α1 AMPK by low intensity contraction in the epitrochlearis muscle (Toyoda *et al* 2006). Both isoforms are deactivated similarly by PP2C but α2 is deactivated by PP2A to a much greater extent than α1 (Salt *et al* 1998). α1 and α2 AMPK may also have distinct physiological functions. It has been shown only α2 AMPK co-purifies with heart ACC (Dyck *et al* 1999) suggesting a role for this isoform in the regulation of cardiac ACC. α2 AMPK has also been suggested to be the isoform involved in the regulation of glucose homeostasis (Jorgensen *et al* 2004). A selective activation of α2 has been also observed in skeletal muscle in response to leptin (Section 1.6.5.i). Both α1 and α2 AMPK are found in the cytosol but α2 AMPK is also found in the nucleus in INS-1 cells and CCL13 cells (Salt *et al* 1998). This localisation is not dependent on the activation state of α2 or association with the β and γ subunits (Salt *et al* 1998).

The β subunit binds both α and γ subunits and is thought to act as a scaffold protein. There are two isofoms of the β subunit; β1 (38kDa) and β2 (34kDa) (Thornton *et al* 1998). The two isoforms display similar C-terminal sequence but show less similarity at the N-terminal sequence (Thornton *et al* 1998). AMPK complexes containing different β subunits do not show differences in activity and there is no evidence of preferential association of the two β isoforms with different α isoforms *in vitro* (Thornton *et al* 1998). Both isoforms are generally distributed. The liver expresses mainly β1 and a small amount of β2, while the opposite pattern is seen in skeletal muscle (Thornton *et al* 1998). The heart expresses both isoforms but β2 is slightly more abundant than β1 (Thornton *et al* 1998). The yeast homologues of the β subunit contain conserved domains termed KIS and ASC, which are involved in the

association with Snf1p and Snf4p, respectively (Yang *et al* 1994; Jiang and Carlson 1997). Putative KIS and ASC domains are also found in the AMPK β subunits at the internal region and the C-terminal region, respectively (Gao *et al* 1996; Woods *et al* 1996; Thornton *et al* 1998). However, in contrast to SNF1, a truncation study has revealed that the β subunit binds both α and γ subunits *via* a C-terminal sequence (residues 186-270 for β1) (Iseli *et al* 2005). The C-terminal residues 241-270 have also been shown to be sufficient to bind the γ subunit, but not the α subunit (Iseli *et al* 2005). The KIS domain in the β subunit is not required for the association with the α or γ subunit (Hudson *et al* 2003). The internal region (amino acids 68-163) of the β subunits which overlaps with the putative KIS domain has been identified as the glycogen binding domain (GBD) closely related to the isoamylase domain found in glycogen and starch branching enzymes (Polenkhina *et al* 2003). The association of AMPK with glycogen *in vivo* has been suggested by the co-localisation of the β subunits with glycogen phosphorylase and synthase (Polenkhina *et al* 2003; Hudson *et al* 2003). The GBD domain is likely to be important for the regulation of glycogen metabolism by AMPK (Section 1.6.5.k). The β subunits are myristoylated at the N-terminus (Thornton *et al* 1998). The myristoylation is involved in the membrane association of AMPK (Mitchelhill *et al* 1997; Warden *et al* 2001). The removal of the myristoylation has been also shown to increase AMPK activity by a mechanism independent of a change in the Thr172 phosphorylation (Warden *et al* 2001).

The γ subunit contains two pairs of CBS (cystathione-β-synthase) domains (CBS1/2 and CBS3/4) called the Bateman modules which are proposed to bind AMP and ATP (Kemp 2004; Adams *et al* 2004). The involvement of the CBS domains in AMP binding is supported by the observations that some point mutations in these domains make AMPK insensitive to AMP (Hamilton *et al* 2001; Adams *et al* 2004). Moreover, several naturally occurring mutations in the human γ2 subunit (R302Q, H383R, and R531G), which are associated with the development of the Wolff-Parkinson-White syndrome, are found within the CBS domains, and these mutations have been shown to decrease the sensitivity of AMPK to AMP (Daniel and Carling 2002a). The inhibition of the stimulatory effect of AMP on AMPK by ATP is thought to be due to the competitive binding of ATP to the Bateman modules (Adams *et al* 2004). There are three isoforms of the γ subunit; γ1, γ2, and γ3. The γ2 gene has two transcriptional start sites and two forms (short and long) of γ2 are

predicted (Cheung *et al* 2000). The γ2 contains a putative nuclear localisation signal (Cheung *et al* 2000). The AMPK complexes containing different γ subunits show differences in the dependence for AMP for activation. The AMPK with γ2 shows the highest AMP dependence followed by γ1 and γ3 (Cheung *et al* 2000). There has been no conclusive evidence of selective association between the three γ isoforms and the α isoforms (Cheung *et al* 2000), although the possibility of preferential association of γ3 with α2 and β2 in the skeletal muscle has been reported (Mahlapuu *et al* 2004). In most tissues, except for the brain and testis, the AMPK complex containing γ1 accounts for the majority of the AMPK activity, and the remainder is accounted for by the γ2-containing complex (Cheung *et al* 2000). The heart expresses both γ1, which is the predominant form, and γ2 (Li *et al* 2006). The expression of γ3 is confined to the skeletal muscle (Cheung *et al* 2000; Yu *et al* 2004).

1.6.3 AMPK kinases (AMPKKs)

AMPK is activated by the phosphorylation of Thr172 of the α subunit by AMPKK. The mutation of Thr172 to alanine completely abolishes the activation of AMPK by partially purified AMPKK (Stein *et al* 2000). The identity of the upstream kinase(s) that phosphorylate(s) Thr172 was elusive for a long time. Earlier studies on AMPKK mostly used a partially purified AMPKK fraction from rat liver. Recently, three yeast AMPKKs have been identified (Pak1p, Tos3p and Elm1p) which can phosphorylate the residue equivalent to Thr172 in the activation loop of Snf1p (Hong *et al* 2003; Sutherland *et al* 2003). Subsequently, LKB1 in mammals, which has a catalytic domain related to these three yeast kinases, has been shown to phosphorylate Thr172 and activate AMPK *in vitro* (Hong *et al* 2003; Hawley *et al* 2003; Woods *et al* 2003; Shaw *et al* 2003). LKB1 is known as a serine/threonine kinase which has a tumour suppressor role. Mutation of LKB1 causes the Peutz-Jeghers syndrome (Boudeau *et al* 2003). LKB1 (~50kDa) purified from rat liver is associated with two accessory proteins; STRADα (~45/48kDa) and MO25α (~40kDa) (Hawley *et al* 2003). The heterotrimeric complex of LKB1 is required for its full activity towards AMPK (Hawley *et al* 2003). LKB1 seems to equally activate AMPK complexes containing different subunit isoforms (Woods *et al* 2003). The role of LKB1 as an AMPKK *in vivo* is supported by several observations. First, an anti-LKB1 antibody could remove

most of the AMPKK activity from the partially purified liver AMPKK fraction (Hawley *et al* 2003; Woods *et al* 2003). Second, in cultured cell lines, the inhibition of LKB1 by expression of catalytically inactive LKB1 inhibited the increase in Thr172 phosphorylation induced by AICAR and H_2O_2 (Woods *et al* 2003; Shaw *et al* 2003). Third, in HeLa cells, which lack endogenous LKB1, and in MEF cells from a LKB1 knock out mouse, AICAR or H_2O_2 failed to increase Thr172 phosphorylation (Hawley *et al* 2003; Shaw *et al* 2003). Lastly, in transgenic mice, deletion of LKB1 in the liver or skeletal muscle greatly decreased the basal Thr172 phosphorylation and almost completely abolished the increase in the Thr172 phosphorylation caused by metformin, AICAR, or skeletal muscle contraction (Shaw *et al* 2005; Sakamoto *et al* 2005). The heart from the transgenic mice lacking cardiac expression of LKB1 also showed almost complete abolition of the basal activity of α2 AMPK and a moderate reduction in the activity of α1 AMPK, indicating a significant role of LKB1 in cardiac AMPK regulation (Sakamoto *et al* 2006).

Despite the lack of LKB1, a small amount of Thr172 phosphorylation could still be detected in HeLa cells and MEF cells from LKB1 knock-out mice (Hawley *et al* 2003). This was not due to non-phospho specific binding of the anti-Thr172 antibody as it was almost completely abolished by incubation with PP2A (Hawley *et al* 2005). Moreover, a Ca^{2+} inophore was shown to increase Thr172 phosphorylation and AMPK activity in HeLa cells. These observations prompted the search for AMPKK other than LKB1. The AMPKK activity towards recombinant AMPK present in a HeLa cell lysate could be completely abolished by STO-609, an inhibitor of CAMKKα and β (Hurley *et al* 2005). The effect of a Ca^{2+} inophore to activate AMPK in the HeLa cells was also inhibited by STO-609 or by small interfering RNA (siRNA) against CAMKKα or β, although the inhibitory effect of siRNA against CAMKKβ was much greater than that for CAMKKα (Hurley *et al* 2005; Hawley *et al* 2005; Woods *et al* 2005). Both CAMKKα and β can phosphorylate Thr172 and activate recombinant AMPK *in vitro*, but CAMKKβ activates AMPKK more readily than CAMKKα (Hawley *et al* 2005; Woods *et al* 2005). These observations strongly suggest that in addition to LKB1, CAMKKβ and possibly CAMKKα also act as AMPKKs *in vivo*. In brain slices, the activation of AMPK by depolarlisation or an increase in Ca^{2+} induced by increasing K^+ concentration has been shown to be inhibited by STO-609, suggesting a role for CAMKKs in the regulation of AMPK in

this organ (Hawley *et al* 2005). However, although CAMKKα and β are highly expressed in the brain, they are poorly expressed in other tissues (Anderson *et al* 1998). The heart has been reported to express CAMKKβ but not CAMKKα (Allard *et al* 2006). The role of CAMKKβ in the regulation of cardiac AMPK remains to be determined.

There still remains the possibility of AMPKKs different from LKB1 or CAMKKs. The possibility of a yet unidentified AMPKK that is activated during ischemia has been reported in the heart (Section 1.6.5.o). Suzuki *et al* have demonstrated that in several cultured cell lines, including the human pancreatic cancer cell line (PANC-1), HeLa and human fibroblast cell lines, AMPK Thr172 phosphorylation is increased by incubation with growth factors such as IGF-1 (Suzuki *et al* 2004). The tyrosine phosphorylation of ATM (ataxia telangiectasia mutated) appears to be involved in this effect as the activation of AMPK was blocked by genistein or antisense mRNA for ATM (Suzuki *et al* 2004). The ATM immunoprecipitated from the IGF-1-stimulated cells phosphorylated the AMPK α catalytic domain *in vitro*, suggesting ATM may be directly upstream of AMPK (Suzuki *et al* 2004).

1.6.4 Phosphorylation sites other than Thr172

In addition to Thr172, AMPK is known to be phosphorylated at several sites on the α and β subunits (Figure 1.3). The α subunit has been shown to be phosphorylated at two other sites, Thr258, and Ser485 (α1)/Ser491 (α2) (Woods *et al* 2003). The mutation of Thr258 or Ser485/491 to asparatic acid to mimic phosphorylation did not affect the kinase activity or sensitivity of AMPK to AMP (Woods *et al* 2003). Using constitutively active and catalytically inactive mutants of AMPK, these sites were shown to be phosphorylated by both autophosphorylation and by a partially purified AMPKK (Woods *et al* 2003). However, the upstream kinases that phosphorylate Thr258 and Ser485/491 were thought to be different from that which phosphorylates Thr172 because the sequences surrounding the Thr258 and Ser485/491 were similar to each other but different from the sequence surrounding the Thr172 (Woods *et al* 2003). Unlike Thr172, the Ser485 phosphorylation was also resistant to incubation with PP2C, PP1 or PP2A (Woods *et al* 2003). Recently, Horman *et al* have

confirmed that Thr258 and Ser485 are autophosphorylated but they are not phosphorylated by LKB1 (Horman *et al* 2006). Moreover, PKB has been shown to phosphorylate recombinant α1β1γ1 and α2β1γ1 AMPK *in vitro* at Ser485/491. The phosphorylation of Ser485 did not directly affect the kinase activity but *in vitro* preincubation of AMPK with PKB and phosphorylation of Ser485 decreased the Thr172 phosphorylation and the activation of AMPK by LKB1, suggesting a hierarchical mechanism where the prior phosphorylation of Ser485 inhibits the subsequent phosphorylation at Thr172 (Horman *et al* 2006).

The β1 subunit has been reported to be phosphorylated at Ser24, Ser25, Ser96, Ser101, Ser108, and Ser182 (Mitchelhill *et al* 1997; Woods *et al* 2003; Chen *et al* 1999). The phosphorylations of Ser24/25 are mutually exclusive (Mitchelhill *et al* 1997). The only reported phosphorylation on the β2 subunit is Ser182 (Chen *et al* 1999). Phosphorylation of these residues may regulate the AMPK activity, cellular localisation, or interaction with substrate. Warden *et al* showed that mutation of Ser108 to alanine decreased AMPK activity while mutation of Ser24/25 or Ser182 did not affect the activity but increased the nuclear localisation (Warden *et al* 2001). Ser108 is within the GBD and mutating it to glutamic acid has been shown to partially inhibit the association of GBD with glycogen (Polekhina *et al* 2003). Most of the β phosphorylation sites identified are autophosphorylation sites (Mitchelhill *et al* 1997; Woods *et al* 2003). However Ser182 and some of the other sites are also likely to be phosphorylated by separate kinases (Mitchelhill *et al* 1997). The catalytically inactive mutant of AMPK has been reported to be phosphorylated at the β subunit by a partially purified AMPKK (Mitchelhill *et al* 1997; Stein *et al* 2000) although this was not observed by other investigators (Woods *et al* 2003). This discrepancy is likely to be due to the difference in the composition of the partially purified AMPKK used in these studies. Recently, incubation of recombinant AMPK with PKB has been shown to increase phosphate incorporation into the β1 but not β2 subunit (Horman *et al* 2006). The significance of PKB phosphorylation of the β subunit in the regulation of AMPK remains to be determined.

1.6.5 The regulation of AMPK by AMP/ATP and other factors

(a) AMP/ATP

In vitro, AMP activates AMPK by a direct allosteric mechanism and by increasing the phosphorylation of AMPK at Thr172 by AMPKK. This phosphorylation is the key event which accounts for most of the activation of AMPK by AMP. Recent studies in cultured cells and in skeletal muscle lacking LKB1 expression have suggested that LKB1 is required for the phosphorylation of AMPK by treatments that increase the AMP/ATP ratio (Section 1.6.3). However, it has been shown that AMP does not directly activate LKB1, although the phosphorylation of AMPK by LKB1 is stimulated in the presence of AMP (Hawley *et al* 2003; Woods *et al* 2003; Shaw *et al* 2003). The activity of LKB1 purified from cultured cells or from skeletal muscle treated with agents that increase AMP/ATP is also not different from that in untreated cells (Woods *et al* 2003; Shaw *et al* 2003; Sakamoto *et al* 2004). LKB1 is known to be phosphorylated at up to eight residues (Boudeau *et al* 2003), and LKB1 immunoprecipitated from a liver AMPKK fraction exists as several species which seem to represent different phosphorylation states of LKB1 (Woods *et al* 2003). However, none of the phosphorylations of LKB1 appear to regulate LKB1 catalytic activity (Sapkota *et al* 2001 and 2002). Consistent with this, preincubation of LKB1 with PP1, PP2A or PP2C does not affect the phosphorylation of AMPK by LKB1 (Woods *et al* 2003). These observations suggest that LKB1 is constitutively active and AMP stimulates the Thr172 phosphorylation by making AMPK a better substrate for LKB1. The exact molecular mechanism for this effect is unknown. According to the model suggested by Cheung *et al*, AMP binding to both α and γ subunits stabilises the conformation where the autoinhibitory region of the α subunit is removed from the catalytic site, exposing Thr172 (Cheung *et al* 2000). Alternatively, Adams *et al* has proposed that ATP binding to the CBS domains of the γ subunit positions a part of the ATP molecule in the activation loop region of the α subunit and blocks the phosphorylation of Thr172 (Adams *et al* 2004). AMP binding has been suggested to stimulate the Thr172 phosphorylation by displacing ATP and removing this inhibitory interaction (Adams *et al* 2004). The constitutive activity and insensitivity of LKB1 to AMP is consistent with the observations made with the total

AMPKK activity present in the INS-1 cell lysate (Hamilton *et al* 2002). However, an earlier study by Hawley *et al* showed that AMPKK partially purified from the liver was directly activated by AMP (Hawley *et al* 1995). Although LKB1 appears to be the predominant AMPKK in the liver, the possibility of as yet unidentified AMPKK(s) which is/are activated by AMP can not be ruled out.

Whether CAMKK is involved in the phosphorylation of AMPK in response to an increase in AMP/ATP is not clear. CAMKKα and β are activated by Ca^{2+}/calmodulin but they are not directly activated by AMP (Hawley *et al* 2005; Woods *et al* 2005). This observation alone does not rule out the possibility that AMP facilitates the phosphorylation of Thr172 by CAMKKβ which has been reported to have some activity in the absence of stimulation by Ca^{2+}/calmodulin (Anderson *et al* 1998). However, unlike LKB1, AMP has been shown to have no effect on the *in vitro* phosphorylation of AMPK by CAMKKs (Hawley *et al* 2005). Although this observation contradicts the earlier study by the same group which showed a stimulatory effect of AMP (Hawley *et al* 1995), it raises the possibility that the AMP-induced facilitation of Thr172 phosphorylation by LKB1 may involve a specific interaction between LKB1 and AMPK. Several other observations suggest that CAMKKs specifically mediate the phosphorylation of Thr172 in response to Ca^{2+}. In HeLa cells, which lack LKB1, AMPK is not activated by AICAR or H_2O_2 but it is activated by a Ca^{2+} inophore (Hurley *et al* 2005; Hawley *et al* 2005). In brain slices, STO-609 inhibited the activation of AMPK by Ca^{2+} but it did not affect the activation of AMPK by phenformin, which increased cellular AMP/ATP (Hawley *et al* 2005). In contrast to these studies, Woods *et al* showed that Thr172 phosphorylation could be increased by H_2O_2 in HeLa cells and in LKB1-deficient MEF cells (Woods *et al* 2005). Moreover, transfection of HeLa cells with siRNA against CAMKKβ decreased the basal AMPK activity and both H_2O_2 and inomycin stimulated AMPK activity (Woods *et al* 2005). They also showed that in NIH3T3 cells, which express both LKB1 and CAMKKs, the activation of AMPK by inomycin, AICAR and H_2O_2 were only partially inhibited by STO-609 whereas in the LKB1-deficient MEF cells, their effects were completely inhibited by the inhibitor, suggesting that Ca^{2+} and AMP activate AMPK *via* both LKB1 and CAMKKβ (Woods *et al* 2005). Offering a possible explanation for the discrepancy between this study and those by Hurley *et al* and Hawley *et al* described above, these

authors suggested that although LKB1 and CAMKKβ are likely to be responsible for the activation of AMPK by AMP and Ca^{2+} signalling, respectively, the distinction is likely to be blurred *in vivo* because an increase in Ca^{2+} is often associated with an increase in AMP and *vice versa*, possibly due to the action of the Ca^{2+} pump (Woods *et al* 2005).

(b) Creatine/Creatine phosphate (Cr/CrP)

AMPK has been reported to be inhibited *in vitro* by physiological concentrations of creatine phosphate (CrP) (5-40mM), and this effect has been shown to be antagonised by creatine (Cr) (Ponticos *et al* 1998). The regulation of AMPK activity by Cr/CrP is consistent with the energy sensing role of AMPK. The effect of Cr/CrP on AMPK is not affected by the presence of AMP, suggesting Cr/CrP bind to a site distinct from the AMP binding site (Ponticos *et al* 1998). Whether Cr/CrP affects the phosphorylation of Thr172 by AMPKKs remained unknown. Recently, Taylor *et al* showed that physiological concentrations of CrP (5-50 mM) did not affect the activation of recombinant α1β1γ1 or α2β2γ2 by the recombinant LKB1 complex or the activation of AMPK purified from the liver by recombinant LKB1 (Taylor *et al* 2005). Moreover, the activity of α1β1γ1 or α2β2γ2 were not inhibited by CrP, and Cr did not activate α2β2γ2 or the activation of α2β2γ2 by recombinant LKB1 (Taylor *et al* 2005). However, they did observe an inhibitory effect of CrP on crude AMPK purified from rat liver and skeletal muscle by PEG precipitation. However, this effect was not specific for CrP (glucose-6-phosphate also inhibited crude AMPK), and the inhibitory effect by CrP or glucose-6-phosphate was not seen in the purer preparation of AMPK obtained by immunoprecipitation, suggesting that the inhibitory effect of CrP seen in the crude AMPK preparation and possibly that observed by Ponticos *et al* may be an artefact due to contaminating enzymes such as creatine kinase and phosphatases that act on CrP or other small phosphomolecules and possibly perturb the concentrations of free phosphate, ATP or AMP (Taylor *et al* 2005).

(c) NAD^+/NADH

The recombinant AMPK heterotrimer has been shown to be activated by β-NAD^+

and inhibited by β-NADH *in vitro* (Rafaeloff-Phail *et al* 2004). This effect depends on the concentrations of AMP and ATP; increasing the concentration of AMP increased the potency of NADH to inhibit AMPK and slightly increased that of NAD^+ to activate AMPK (Rafaeloff-Phail *et al* 2004). The ability of NADH to inhibit AMPK is decreased with the increasing concentration of ATP (Rafaeloff-Phail *et al* 2004).The physiological significance of this effect is not clear. The effect of NAD^+/NADH on covalent modification of AMPK is unknown.

(d) Hyperosmotic stress

In H-2Kb muscle cells, sorbitol caused activation of both α1 and α2 AMPK and increased the Thr172 phosphorylation (Fryer *et al* 2002). The effect of sorbitol on the AMPK activity was independent of changes in the AMP/ATP ratio and was additive to the effects of AICAR and DNP, suggesting hyperosmotic stress activates AMPK by a distinct pathway (Fryer *et al* 2002). This is supported by the study by Daniel and Carling who showed that the α1 chimeric protein containing the C-terminal regulatory region of snf1p coexpressed with the SNF1 regulatory subunits in CCL13 cells was not activated by an increase in the AMP/ATP ratio but was activated by sorbitol (Daniel and Carling 2002b). LKB1 was shown to be required for the effect of sorbitol to increase the Thr172 phosphorylation (Woods *et al* 2003, Shaw *et al* 2003). However, like AMP/ATP-raising treatments, sorbitol did not affect the LKB1 activity in cultured cells (Woods *et al* 2003, Shaw *et al* 2003). Thus, how sorbitol, which does not increase AMP/ATP, increases the LKB1-mediated Thr172 phosphorylation is unknown. Hyperosmotic stress is known to activate a number of kinases including PKA, MLTK, PI3K, PKC, and p38 MAPK (Mao *et al* 2004; van der Kaay *et al* 1999; Schaffler *et al* 2000). It appears that PI3K, PKC, and p38 MAPKα/β are not involved in the sorbitol induced AMPK activation (Fryer *et al* 2002).

(e) Insulin

Insulin has been known to inhibit AMPK activity in the heart (Gamble and Lopaschuk 1997; Clark *et al* 2004). This effect was shown to be independent of

changes in the AMP/ATP ratio (Beauloye *et al* 2001). Moreover, insulin markedly decreased the extent of the AMPK activation and Thr172 phosphorylation increased by ischemia or anoxia by a mechanism which was again independent of AMP/ATP and Cr/CrP but was sensitive to wortmannin (Beauloye *et al* 2001). Subsequently, Kovacic *et al* demonstrated that the Thr172 phosphorylation was decreased in the heart of transgenic mice and neonatal cardiac myocytes over-expressing constitutively active PKB (Kovacic *et al* 2003). A possible mechanism by which insulin inhibits AMPK activity has been recently described by Horman *et al*, who showed that PKB phosphorylates the α subunit of AMPK at Ser485/491 *in vitro* (Horman *et al* 2006) (Section 1.6.4). They also showed in the perfused heart that the phosphorylation of Ser485/491 was increased by insulin and by ischemia. The phosphorylation of Ser485/491 by ischemia is consistent with the autophosphorylation of this site. It may be possible that this autophosphorylation serves as a negative feedback mechanism. Similar to the *in vitro* observation, the prior phosphorylation of Ser485/491 by insulin in the perfused heart prevented the phosphorylation of Thr172 by ischemia (Horman *et al* 2006). Consistent with Horman *et al*, Soltys *et al* showed that adenoviral expression of constitutively active PKB in neonatal cardiac myocytes prevented the increase in the phosphorylation of Thr172 and ACC2 Ser227 (ACC1 Ser79) induced by adenoviral expression of LKB1 or chemical hypoxia (Soltys *et al* 2006).

(f) Metformin

The anti-diabetic drug metformin decreases plasma glucose by inhibiting hepatic glucose production and by stimulating peripheral glucose utilisation. Metformin increases Thr172 phosphorylation and activates AMPK in various tissues including the liver (Zhou *et al* 2001), skeletal muscle (Musi *et al* 2002), heart (Kovacic *et al* 2003), adipocyte (Huypens *et al* 2005b) and endothelial cells (Zou *et al* 2004). Metformin has been shown to inhibit complex 1 of the mitochondrial respiratory chain (El Mir *et al* 2000; Owen *et al* 2000) and the activation of AMPK by metformin is associated with decreased ATP and CrP in skeletal muscle (Musi *et al* 2002). However, AMP/ATP-independent increases in Thr172 phosphorylation and AMPK activity have been reported in CHO fibroblasts, a rat hepatoma cell line

(H4IIE), and in cultured muscle cells (H-2Kb) (Hawley *et al* 2002; Fryer *et al* 2002). Metformin did not directly stimulate the activation of purified AMPK by a partially purified AMPKK *in vitro* and it did not protect AMPK from dephosphorylation by PP2C (Hawley *et al* 2002). However, the AMPKK activity present in the lysate of metformin-treated H4IIE cells was increased compared to that in untreated cells (Hawley *et al* 2002), raising the possibility that metformin activates AMPKK through a covalent modification.

(g) Peroxynitrite

A series of studies by Zou *et al* primarily done in endothelial cells suggested that some of the reported AMP/ATP-independent activation of AMPK, including metformin and hypoxia (Section 1.6.5.o), may be mediated by peroxynitrite (Zou *et al* 2002, 2003, and 2004; Xie *et al* 2006). In endothelial cells, both metformin and peroxynitrite increased Thr172 phosphorylation and activated AMPK without changing AMP/ATP (Zou *et al* 2003 and 2004). Peroxynitrite has been shown to be increased by metformin in endothelial cells *in vitro* or in the heart of metformin-administered mice (Zou *et al* 2004). The involvement of peroxynitrite in the activation of AMPK by metformin in endothelial cells is suggested by the observation that the activation of AMPK by metformin was abolished by inhibiting the two precursors of peroxynitrite, superoxide and nitric oxide, by over-expression of superoxide dismutase and by an eNOS inhibitor, respectively (Zou *et al* 2004). Metformin is likely to increase superoxide by inhibiting the mitochondrial complex I as functional mitochondria were required for the effect of metformin to produce superoxide, and rotenone, another inhibitor of the complex I, also increased superoxide (Zou *et al* 2004). In endothelial cells, hypoxia or reoxygenation after hypoxia caused the activation of AMPK and this effect was inhibited by overexpression of superoxide dismutase or a NOS inhibitor, indicating that peroxynitrite is also involved in the regulation of AMPK during hypoxia (Zou *et al* 2003). Further limited studies by the same investigators have shown that peroxynitrite activates AMPK in other cell types including cardiac myocytes, retinal pericytes, cultured rat vascular smooth muscle cells and mouse 3T3-L1 pre-adipocytes, suggesting the possibility that the regulation of AMPK by metformin and

hypoxia in these cells may also involve peroxynitrite (Zou *et al* 2004; Xie *et al* 2006). Supporting this possibility, the effect of metformin to increase Thr172 phosphorylation in the cardiac myocytes was inhibited by superoxide dismutase and by a NOS inhibitor (Zou *et al* 2004).

These investigators have also provided evidence that peroxynitrite activates AMPK by increasing the association of LKB1 with the AMPK α subunit (Xie *et al* 2006). This is a novel mechanism for regulating the 'effective activity' of LKB1, which appears to be constitutively active. They have demonstrated that PKCζ, which has been shown to be activated by peroxynitrite, is required for the association of LKB1 with AMPK (Xie *et al* 2006). Moreover, PKCζ has been shown to phosphorylate LKB1 at Ser428 (Ser431 in the human sequence) *in vitro*, and the mutation of this residue to alanine inhibited the peroxynitrite-induced activation of AMPK, suggesting that Ser428 may be important for the interaction of LKB1 with AMPK (Xie *et al* 2006). Previously, the phosphorylation of Ser428 has been reported to have no effect on the catalytic activity of LKB1 (Sapkota *et al* 2001). The possible role of Ser428 in the regulation of the interaction of LKB1 with AMPK may explain the previous observation reported by Sapkota *et al* that mutating Ser428 prevented the suppression of cell growth by LKB1 in G361 melanoma cells despite the lack of the effect of the Ser428 mutation on the catalytic activity of LKB1 (Sapkota *et al* 2001). It may also explain the observation made by Kimball *et al* that the increase in the AMPK Thr172 phosphorylation in response to glucagon in the liver was associated with the increased PKA phosphorylation of LKB1 at Ser428 (Kimball *et al* 2004). In the heart, ischemia has been reported to activate AMPK by a PKC-dependent mechanism (Nishino *et al* 2004). Although the activation of PKCε by ischemia was demonstrated, the activation of PKCζ was not determined in this study (Nishino *et al* 2004). Whether, PKC isoforms other than PKCζ can phosphorylate LKB1 Ser428 and regulate AMPK is unknown.

PKCζ was suggested to be activated by peroxynitrite through a cSrc-PI3K-PDK1 pathway in endothelial cells because the activation of AMPK by peroxynitrite was inhibited by wortmannin or by expression of dominant negative cSrc or PDK1 (Zou *et al* 2003). The activation of PI3K by peroxynitrite contradicts the observation that insulin inhibits AMPK *via* the PI3K-PKB pathway (Section 1.6.5.e). However, it

may be possible that peroxynitrite activates different isoforms or different pools of PI3K.

(h) Ca^{2+}

In addition to the aforementioned activation of AMPK by Ca^{2+} in brain slices and cultured cells (Section 1.6.5.a), stimulation of several cell surface receptors have also been reported to activate AMPK by increasing intracellular Ca^{2+}. The stimulation of the thrombin receptor or P2Y purinergic receptor in the human umbilical vein endothelial cells (HUVEC) and triggering of the T cell antigen receptor in T lymphocytes have all been shown to increase Thr172 phosphorylation *via* AMP/ATP-independent and Ca^{2+}-dependent mechanisms (Stahmann *et al* 2006; da Silva *et al* 2006; Tamas *et al* 2006). The increase in Ca^{2+} was likely to be mediated by G_q and PLC (Stahmann *et al* 2006). The effects of all three receptors on the AMPK activation were abolished by STO-609, indicating the involvement of CAMKKs (Stahmann *et al* 2006; da Silva *et al* 2006; Tamas *et al* 2006). LKB1, which is also expressed in HUVEC, does not seem to be involved in the activation of AMPK by thrombin as the effect was insensitive to siRNA against LKB1 (Stahmann *et al* 2006). In contrast, the activation of AMPK in HUVEC by AICAR was insensitive to CAMKKβ inhibition but it was inhibited by siRNA against LKB1 (Stahmann *et al* 2006). These observations provide additional support for distinct roles of LKB1 and CAMKKs. Histamine in HUVEC has also been shown to activate AMPK *via* Ca^{2+}-dependent mechanism (Thors *et al* 2004). Stimulation of α_1 adrenergic receptor in skeletal muscle also appears to activate AMPK by a Ca^{2+}-dependent mechanism (Hutchinson and Bengtsson 2006; Section 1.6.5.p).

(i) Adipokines

An adipocyte-derived hormone leptin has been shown to have different effects on AMPK in different tissues. Leptin activates α2 but not α1 AMPK in the skeletal muscle (Minokoshi *et al* 2002). However, leptin inhibits AMPK in the hypothalamus (Andersson *et al* 2004; Minokoshi *et al* 2004), while it seems to have no effect on AMPK in the perfused heart (Atkinson *et al* 2002). *In vivo* administration of leptin

has been shown to activate AMPK in skeletal muscle *via* two pathways; the direct effect of leptin on the skeletal muscle and indirect slower effect through the hypothalamic-sympathetic nervous system (Minokoshi *et al* 2002). The direct effect of leptin seems to be mediated by the AMP-dependent pathway as a transient increase in AMP was observed immediately after leptin administration (Minokoshi *et al* 2002). However, the delayed indirect effect is likely to be mediated *via* an AMP/ATP-independent mechanism through the α_1 adrenergic receptor as the *in vivo* effect was sensitive to phentolamine and phenylephrine activated AMPK in isolated skeletal muscle (Minokoshi *et al* 2002).

Another adipocyte-derived hormone adiponectin activates AMPK in skeletal muscle, liver, and pancreatic β cells (Yamauchi *et al* 2002; Huypens *et al* 2005a). Adiponectin receptors are also expressed in the cardiac myocytes, which also have been shown to secrete adiponectin (Pineiro *et al* 2005). Adiponectin increased AMPK Thr172 phosphorylation in the HL-1 atrial cardiac myocyte cells (Pineiro *et al* 2005). The mechanism by which adiponectin activates AMPK is not clear. However adiponectin increased AMP in skeletal muscle, suggesting the involvement of the AMP-dependent pathway (Yamauchi *et al* 2002). In bovine aortic endothelial cells, the adiponectin-induced increase in AMPK Thr172 phosphorylation was inhibited by wortmannin, suggesting that the adiponectin effect may also involve PI3K (Chen *et al* 2003).

Another adipocyte-derived hormone resistin inhibits AMPK in the liver and skeletal muscle by an unknown mechanism (Banerjee et al 2004; Muse et al 2004; Palanivel and Sweeney 2005).

(j) Ghrelin

Ghrelin is produced in the stomach and it stimulates food intake. Like leptin, ghrelin has different effects on AMPK in different tissues. Ghrelin increases Thr172 phosphorylation or AMPK activity in the hypothalamus and heart (Andersson *et al* 2004; Kola *et al* 2005). However, ghrelin decreases the Thr172 phosphorylation or AMPK activity in the liver and adipose tissue, while it has no effect in skeletal

muscle (Kola *et al* 2005 and Barazzoni *et al* 2005). The mechanism of this tissue specificity is unknown.

(k) Glycogen

There appears to be a complex relationship between AMPK activity and the cellular glycogen content. High cellular glycogen was shown to be associated with decreased basal and AICAR-stimulated α2 AMPK activity in skeletal muscle (Wojtaszewski *et al* 2002). Moreover, the activation of AMPK in skeletal muscle induced by AICAR or by glucose withdrawal was accompanied by a decrease in the glycogen synthase activity (Wojtaszewski *et al* 2002; Halse *et al* 2003). These observations suggest a reciprocal relationship between AMPK and the cellular glycogen level. The inactivation of glycogen synthase is probably due to the direct phosphorylation of glycogen synthase by AMPK, which has been shown *in vitro* (Carling and Hardie 1989). The mechanism by which glycogen inhibits AMPK is not clear. It has been shown that glycogen does not directly inhibit AMPK activity (Polekhina *et al* 2003). The inhibitory effect may involve glycogen-associated phosphatases or other glycogen-associated enzymes. However, this reciprocal relationship between AMPK and glycogen has not been observed under some conditions. Aschenbach *et al* demonstrated that *in vivo* AICAR administration increased glycogen in skeletal muscle despite the activation of α2 AMPK (Aschenbach *et al* 2002). These authors attributed the glycogen accumulation to increased glucose uptake because no change in the activity of glycogen synthase or phosphorylase was observed (Aschenbach *et al* 2002). Several naturally occurring mutations in the human γ2 gene have been shown to cause excessive glycogen accumulation the heart. The myocardial glycogen accumulation causes ventricular pre-excitation, the condition called Wolff-Parkinson-White syndrome, which is associated with the development of cardiac hypertrophy. Transgenic mice expressing some of these mutations (N488I, R302Q, R531G) have been generated (Arad *et al* 2003; Davies *et al* 2006; Sidhu *et al* 2005). All of these mice showed glycogen accumulation in the heart and cardiac hypertrophy (Arad *et al* 2003; Davies *et al* 2006; Sidhu *et al* 2005). The AMPK activity is decreased in the heart of R302Q-expressing mice (Sidhu *et al* 2005), consistent with the reciprocal relationship between AMPK and glycogen. The cardiac

AMPK activity of the mice expressing the R531G mutation was found to be not different at 1 week of age and there was also no glycogen accumulation at this age (Davies *et al* 2005). However, glycogen accumulation was seen at four week of age and this was associated with the decreased AMPK activity (Davies *et al* 2005). In contrast to these mutations, the N488I mutation is associated with accumulation of glycogen despite increased AMPK activity (Arad *et al* 2003). Thus, the precise relationship between AMPK and glycogen is not clear and requires further research.

(l) Glucose

Glucose has been shown to decrease Thr172 phosphorylation and AMPK activiy in the pancreatic β-cells and skeletal muscle (da Silva Xavier *et al* 2000 and 2003; Itani *et al* 2003; Halse *et al* 2003). While glucose caused the expected decrease in the AMP/ATP ratio in the β-cells (this is implicated in the 'glucose sensing' function of the β-cells and the regulation of insulin release) (da Silva Xavier *et al* 2003)), glucose did not affect the total adenine nucleotides and CrP in skeletal muscle (Itani *et al* 2003). The mechanism by which glucose inhibits AMPK in the skeletal muscle is unknown. However, the possibility of a localised change in free AMP and ATP cannot be ruled out. The effect of glucose does not appear to involve glycogen, because inhibiting glycogen phosphorylase inhibited the decrease in glycogen caused by low glucose but it did not inhibit the activation of AMPK by low glucose (Halse *et al* 2003). Gimeno-Alcaniz and Sazz have demonstrated using the yeast two-hybrid system that the interaction of AMPK α2 and γ1 increased when the glucose concentration was lowered (Gimeno-Alcaniz and Sazz 2003). The significance of this effect is unclear but it may be possible that glucose or a glucose metabolite causes a conformational modification of the AMPK complex which may affect the regulation by AMPKK or phosphatases.

(m) Long chain fatty acids

The physiological concentrations of long chain fatty acids (LCFA) such as palmitate have been shown to increase Thr172 phosphorylation and activate AMPK in heart and skeletal muscle (Hickson-Bick *et al* 2000; Clark *et al* 2004; Fediuc *et al* 2006;

Watt *et al* 2006), although this effect has not been observed by some investigators (Olsen and Hansen 2002; Dobrzyn *et al* 2005; Crozier *et al* 2005; Folmes *et al* 2006). Apart from the observation that fatty acids increase Thr172 phosphorylation without a detectable change in AMP/ATP, CrP, or glycogen content (Clark *et al* 2004; Watt *et al* 2006), the mechanism of the fatty acid-induced activation of AMPK is unknown. Watt *et al* reported that paradoxically, the activity of LKB1 towards recombinant AMPK was lower in cells treated with fatty acids than in untreated cells (Watt *et al* 2006). However, they found that palmitate or oleate stimulated the *in vitro* activation of AMPK by LKB1 by a mechanism dependent on the presence of the β and γ subunits of AMPK (Watt *et al* 2006). In contrast to Watt *et al*, Taylor *et al* did not observe a stimulatory effect of palmitate on the activation of AMPK by LKB1 *in vitro* (Taylor *et al* 2005). Moreover, they showed that the phosphorylation and activation of AMPK by LKB1 or AMPKK purified from the liver were inhibited by long chain acyl CoA esters such as palmitoyl CoA *in vitro* (Taylor *et al* 2005). Whether these *in vitro* observations are involved in the regulation of AMPK by fatty acids in intact cells is unknown.

(n) Contraction

The activation of AMPK seen during skeletal muscle contraction has been attributed to the increase in AMP/ATP associated with contraction (Hutber *et al* 1997; Park *et al* 2002). However, Toyoda *et al* recently demonstrated that low intensity contraction of skeletal muscle caused an increase in the Thr172 phosphorylation and activation of α1 AMPK without any changes in the total AMP/ATP (Toyoda *et al* 2006). This could be due to the activation of AMPK by a small or localised change in the free AMP concentration which would not be detected by total cellular adenine nucleotide measurements by HPLC. However, the authors argue that this possibility is unlikely because they did not observe an activation of α2 AMPK, which is more sensitive to AMP than α1 AMPK (Section 1.6.2) during the low intensity contraction. Thus there is the possibility that in addition to AMP/ATP, some other factor is also involved in the activation of AMPK during muscle contraction (Toyoda *et al* 2006). Compared to skeletal muscle, in the heart, the AMP/ATP ratio remains relatively constant during increased contractile activity. However, an increase in the AMP/ATP ratio during

increased contractile activity has been reported in the heart and there have been observations suggesting that increased cardiac contractile activity activates AMPK by increasing the AMP/ATP ratio under some conditions (Section 1.7). Beauloye *et al* have shown that an acute increase in contraction induced by increasing afterload in perfused hearts does not increase AMP or AMPK activity, suggesting cardiac muscle contraction alone (without an increase in AMP) does not activate AMPK (Beauloye *et al* 2002). However, these authors also demonstrated that PKB was activated by increased contraction (Beauloye *et al* 2002). These observations apparently contradict the inhibition of cardiac AMPK by PKB.

(o) Myocardial ischemia

Cardiac ischemia is associated with an increase in AMP/ATP and activation of AMPK through the phosphorylation of Thr172 (Kudo *et al* 1995; Baron *et al* 2005). Sakamoto *et al* studied the role of LKB1 in the activation of AMPK during ischemia using transgenic mice lacking cardiac expression of LKB1 (Sakamoto *et al* 2006). The deletion of LKB1 in the heart prevented the increase in the Thr172 phosphorylation of AMPK in response to ischemia, suggesting that LKB1 mediates the activation of AMPK during ischemia (Sakamoto *et al* 2006). However ischemia has been shown to have no effect on the catalytic activity of LKB1, as measured by *in vitro* phosphorylation of recombinant AMPK by LKB1 immunoprecipitated from the ischemic heart, or on the protein abundance of LKB1, although LKB1 purified from the heart is able to phosphorylate α312 (the truncated catalytic subunit which can be phosphorylated and activated in the absence of AMP), confirming the constitutive activity of LKB1 (Sakamoto *et al* 2006; Altarejos *et al* 2005). Thus, as suggested for the other AMP/ATP-raising conditions, the phosphorylation of Thr172 by LKB1 during ischemia is also likely to be due to the effect of AMP to make AMPK a better substrate for LKB1, rather than a change in LKB1 catalytic activity.

Recent studies suggest that in addition to LKB1, the activation of AMPK during ischemia also involves some other AMPKK which, unlike LKB1, is activated by ischemia. In the aforementioned study by Sakamoto *et al*, whereas the Thr172 phosphorylation of α2 AMPK induced by ischemia was completely abolished,

ischemia could still increase the Thr172 phosphorylation of α1 AMPK in the LKB1-deficient hearts (Sakamoto *et al* 2006). This suggests that LKB1 is the major AMPKK mediating the α2 AMPK activation during ischemia but α1 AMPK is also activated by some other AMPKK, as well as by LKB1 (Sakamoto *et al* 2006). Altarejos *et al* found that although LKB1 can be completely precipitated with 5% PEG6000 and it is not present in the supernatant, AMPKK activity still remained in the supernatant and this AMPKK activity was increased by ischemia (Altarejos *et al* 2005). Moreover, Baron *et al* have shown that the total AMPKK activity present in a heart homogenate is increased by ischemia (Baron *et al* 2005). Given that LKB1 activity is not increased by ischemia, this result further supports the idea that the activation of AMPK during ischemia involves an AMPKK whose catalytic activity is activated by ischemia. Whether this AMPKK is a CAMKK or a yet unidentified AMPKK is unknown. The mechanism by which ischemia activates this AMPKK also remains to be determined. AMP does not appear to activate this AMPKK as the total cardiac AMPKK in the study by Baron *et al* was not directly activated by AMP *in vitro* (Baron *et al* 2005).

Recent studies have revealed that mild ischemia or hypoxia increases Thr172 phosphorylation and AMPK activity without affecting AMP/ATP or Cr/CrP (Altarejos *et al* 2005; Frederich *et al* 2005). This raises the possibility that during severe ischemia, AMPK is activated by two parallel pathways; the AMP pathway and a pathway which is independent of AMP but dependent on some factors that are generated or activated during mild ischemia or hypoxia. Altarejos *et al* observed that the activity of the non-LKB1 AMPKK present in the 5% PEG6000 supernatant was increased by mild ischemia (Altarejos *et al* 2005). Thus, this non-LKB1 AMPKK may be responsible for the activation of AMPK during hypoxia/mild ischemia and for AMP-independent activation of AMPK during severe ischemia. However the involvement of LKB1 in the AMP-independent activation of AMPK during ischemia/hypoxia cannot be ruled out because it is conceivable that 'non-AMP factors' may stimulate the phosphorylation of AMPK by LKB1 by modifying the interaction of AMPK with LKB1 or by modifying a property of AMPK such as the affinity of AMPK for AMP. Supporting the latter possibility, Frederich *et al* have shown that hypoxia increases the sensitivity of AMPK to stimulation by AMP (Frederich *et al* 2005).

(p) Adrenergic receptors

Since the initial report by Moule and Denton that isoproterenol increased Thr172 and AMPK activity in epididymal fat cells (Moule and Denton 1998), several groups have reported the activation of AMPK through adrenergic receptors in the Chinese hamster ovary cells (Kishi *et al* 2000), skeletal muscle (Minokoshi *et al* 2002; Hutchinson and Bengtsson 2006), white adipocytes (Yin *et al* 2003; Daval *et al* 2005) and brown adipocytes (Inokuma *et al* 2005; Hutchinson *et al* 2005). Although these studies did not investigate the signalling mechanism linking the adrenergic receptors to AMPK in great detail, the adrenergic activation of AMPK in adipocytes was shown to be exclusively mediated by the β receptor and the cAMP pathway (Yin *et al* 2003; Daval *et al* 2005; Hutchinson *et al* 2005), while that in skeletal muscle was shown to be exclusively mediated by the α_1 receptor (Hutchinson and Bengtsson 2006). The effect of the α_1 adrenergic stimulation on AMPK in skeletal muscle was also shown to be independent of changes in the AMP/ATP ratio, but it appeared to involve Ca^{2+} (Hutchinson and Bengtsson 2006). It is not clear whether the effect in adipocytes was due to an increase in the AMP/ATP ratio. Inokuma *et al* reported an increase in AMP/ATP after noradrenaline stimulation in brown adipocytes, possibly due to the activation of the uncoupling protein, UCP1 (Inokuma *et al* 2005). However, a subsequent study by Hutchinson *et al* using UCP1 knock-out brown adipocytes showed UCP1 was not necessary for AMPK activation by noradrenaline, although the effect of noradrenaline on AMP/ATP in UCP1-null adipocytes was not determined (Hutchinson *et al* 2005). No measurements of AMP/ATP were made in the other studies reporting adrenergic activation of AMPK in adipocytes. The effect in adipocytes could also be due to β adrenergic stimulation of lipolysis and an increase in long chain fatty acids which have been shown to activate AMPK (Section 1.6.5.m).

1.7 The possibility of the regulation of AMPK through adrenergic receptors in the heart

Clarifying an effect of adrenergic receptor stimulation on the AMPK activity in the heart is important because it may be possible that, given the emerging role of AMPK

as a key regulator of many metabolic processes in the heart (Section 1.6.1), some of the biological effects of adrenergic receptor stimulation are mediated through the regulation of AMPK. This possibility is supported by the recent observations by Hutchinson and Bengtsson that inhibiting AMPK abolished α_1 receptor-mediated glucose uptake in skeletal muscle and partially blocked β receptor-mediated glucose uptake in brown adipocytes (Hutchinson and Bengtsson 2006; Hutchinson *et al* 2005). In the heart, in addition to regulating glucose uptake and fuel metabolism, AMPK is thought to be involved in cardiac hypertrophy and apoptosis, although the precise roles of AMPK in these processes are still not clear (Dyck and Lopaschuk 2006). As summarised in the Figure 1.4, the stimulation of cardiac adrenergic receptors activates a number of pathways that could potentially activate or inhibit AMPK activity. These include the activation of PKA, PKC and Ca^{2+}, which have been shown to activate AMPK in non-cardiac tissues (Sections 1.6.5.g and h), and PKB, which has been shown to inhibit AMPK in the heart (Section 1.6.5.e). The stimulation of adrenergic receptors also increases cardiac muscle contraction which could activate AMPK by the AMP/ATP-dependent pathway. However, An *et al* have reported that isoproterenol has no effect on AMPK Thr172 phosphorylation in perfused hearts or in isolated myocytes (An *et al* 2005). Moreover, Clark *et al* have shown that adrenaline does not change AMPK activity in isolated hearts perfused with glucose as a sole substrate (Clark *et al* 2004). These results suggest that, unlike in skeletal muscle and adipocytes, neither α nor β receptor stimulation directly alters AMPK activity in the heart.

However, the possibility that adrenergic stimulation of contraction activates AMPK through an AMP/ATP-dependent pathway under some conditions can not be ruled out. Studies with perfused hearts have reported different effects of adrenergic stimulation on cardiac AMP/ATP. AMP/ATP is unaffected by adrenergic stimulation in some studies (Collins-Nakai *et al* 1994; Goodwin and Taegtmeyer 1999) and presumably in the aforementioned studies by An *et al* and Clark *et al*, while it is increased in the other studies (Goodwin *et al* 1998b). This is likely to be due to the difference in the intensity of the stimulation, the metabolic substrates included in the perfusion media, or the mode of the perfusion (Langendorff vs. working heart mode). No studies have directly correlated the increase in AMP/ATP during adrenergic

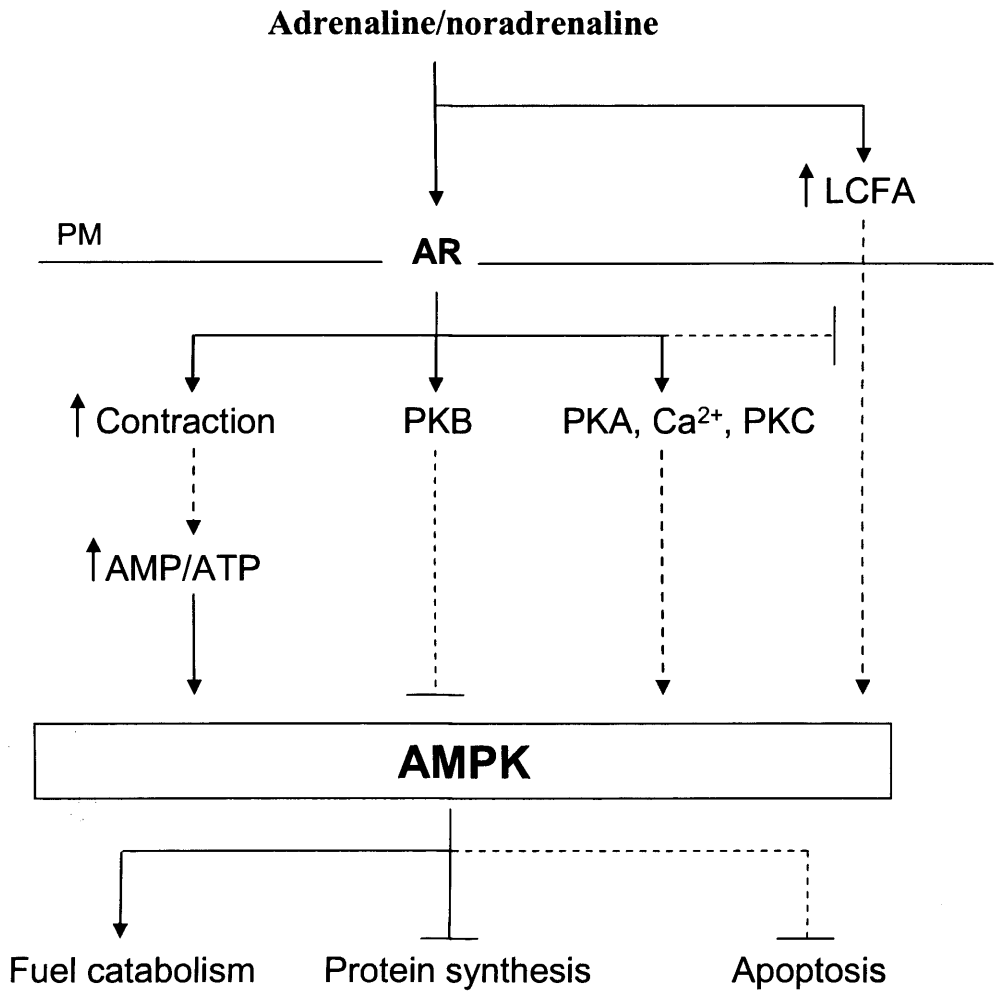


Figure 1.4 The hypothetical regulation of cardiac AMPK by adrenergic agonists

Dotted lines indicate hypothetical pathways based on observations in non-cardiac tissues, or pathways which are still controversial. See text for details.

stimulation to the AMPK activity in perfused hearts. *In vivo* administration of isoproterenol increased AMPK Thr172 phsophorylation in the heart possibly due to the increased heart contraction, although no measurements of AMP/ATP were made (An *et al* 2005). However, other investigators reported that infusion of dobutamine in swine did not cause activation of cardiac AMPK (King *et al* 2005). Again, these differences in *in vivo* observations could be due to the difference in the intensity of contraction. In the former study, the heart was isolated one hour after the isoproterenol injection whereas the heart in the latter study was stimulated for 15 minutes. This possibility is supported by the studies by Coven *et al* and Musi *et al* investigating the effect of exercise, which is known to increase plasma catecholamines (Wadley *et al* 2006), on the activation of AMPK in the heart. Low intensity exercise (10 minutes treadmill running) did not cause activation of cardiac AMPK, while moderate (16 minutes running) and high intensity (32 minutes or 30 minutes running) exercise decreased the cardiac high energy phosphates and caused a graded activation of AMPK in the heart (Coven *et al* 2003; Musi *et al* 2005).

The finding that long chain fatty acids activate AMPK in the perfused heart and in neonatal cardiac myocytes (Section 1.6.5.m) raises the possibility that *in vivo*, catecholamines may activate cardiac AMPK by increasing plasma fatty acids. However, in the aforementioned study by Clark *et al*, it was discovered that although adrenaline did not affect the AMPK activity in the heart perfused with glucose alone, it completely abolished the activation of α2 AMPK (the predominant cardiac isoform (Section 1.6.2)) by palmitate (Clark *et al* 2004). This finding implies that *in vivo*, most of the activation of cardiac AMPK due to the increased supply of fatty acids would be counteracted during adrenergic stimulation. Although detailed information regarding the time-course of the effect of fatty acids to activate AMPK is not available, the effect was not found to be transient in both perfused heart and neonatal myocytes (Clark *et al* 2004; Hickson-Bick *et al* 2000). Thus, given that the heart is constantly exposed to fatty acids, it may be possible that there is a tonic activation of cardiac AMPK by fatty acids *in vivo*. If this turned out to be the case, the adrenergic stimulation *in vivo* would be expected to not only counteract the activation of AMPK by the increased fatty acids supply but also decrease the activity of α2 AMPK in the heart to the level below the unstimulated level. To date, this possibility has not been conclusively examined. Several *in vivo* studies have shown cardiac AMPK is not

inhibited during exercise or in response to infusion of β agonists (Coven *et al* 2003; Musi *et al* 2005; An *et al* 2005; King *et al* 2005). However, the interpretations of some of these studies are complicated by activation of AMPK secondary to an increase in AMP/ATP. In addition, the effect of α_1-receptor stimulation has not been directly examined *in vivo* (e.g. by infusion of α_1 agonists).

The pathway by which fatty acids covalently activate AMPK and how adrenaline inhibits this effect are unknown. The observation that adrenaline only inhibited the activation of α2 AMPK, despite the activation of both α1 and α2 AMPK by palmitate, suggests that adrenergic signalling directly acts on the α2 AMPK complex or the proteins specifically associated with α2 AMPK, rather than blocking the conversion of fatty acids to the metabolite that activates AMPK. The activation of AMPK by fatty acids was found to be independent of changes in AMP/ATP. It is not known whether adrenaline inhibits the activation of AMPK by other conditions that are known to activate AMPK independently of AMP/ATP such as hyperosmotic stress. The activation of cardiac AMPK by high intensity exercise may suggest that adrenaline does not inhibit the activation of AMPK through an increase in AMP/ATP, although this needs to be clarified.

1.8 The purpose of the present study

The purpose of the present study was to investigate further the observation made by Clark *et al* (Clark *et al* 2004) that adrenaline inhibited the increase in AMPK Thr172 phosphorylation and activity induced by palmitate in the heart. Specifically, I sought to elucidate the adrenergic signalling pathways involved in this effect and get some insights into the mechanism by which adrenergic signalling prevents the phosphorylation of AMPK Thr172. I also tried to determine whether this inhibitory effect was specific for the activation of AMPK by long chain fatty acids, or if it could also prevent the activation of AMPK by other conditions such as hyperosmotic stress and AMP-raising treatments. The use of quiescent cardiac myocytes is suited for this purpose for several reasons. First, it allows determination of whether the effect of palmitate and adrenaline observed in perfused hearts was dependent on non-cardiac myocytes (e.g. endothelial cells) in the whole heart. Second, because

myocytes from the same hearts can be incubated under multiple different conditions, effects of different treatments (e.g. inhibitors and activators of adrenergic signalling pathways) on the effect adrenaline on AMPK can be more efficiently investigated than in perfused hearts. Lastly, the use of quiescent cardiac myocytes is suited for the elucidation of the specific effects of adrenaline on different AMPK-activating conditions because it eliminates the possibility of AMPK activation due to an increase in AMP/ATP that might occur during adrenergic stimulation in the contracting heart.

Chapter 2: Materials and Methods

2.1 Commercial preparations

All routinely used chemicals and chemicals which are not specified below were obtained from Sigma-Aldrich or BDH (VWR International).

Purified water used for cardiac myocyte isolation was from BDH (VWR International).

O_2:CO_2 (95%:5%) cylinders were from BOC Ltd.

Sodium pentobarbitone was from Merial.

Collagenase Type 2 and hyaluronidase were from Worthington Biochemical Corporation.

DNase I was from Roche.

(-)-Epinephrine bitartrate, (-)-isoproterenol hydrochloride, (R)-(-)-phenylephrine hydrochloride, phentolamine hydrochloride, (+/-)-propranolol hydrochloride, forskolin, dibutyryl cyclic AMP sodium salt, 8-(4-chlorophenylthio)-2'-O-methyladenosine 3', 5'-cyclic monophosphate (8-CPT-2'-O-Me-cAMP) sodium salt, insulin, wortmannin, D-sorbitol, phenformin hydrochloride, phorbol-12-myristate-

13-acetate, BSA (essentially fatty acid free, fraction V), palmitic acid sodium salt, protein G-Sepharose and bicinchoninic acid protein assay kit were from Sigma-Aldrich.

NEFA C Test kit was from Wako Chemicals Inc.

AICAR, oligomycin, and KN93 were from Calbiochem (Merck Biosciences).

Anti-AMPKα1 antibody (sheep) was from Prof. David Carling, Clinical Sciences Centre, Hammersmith Hospital, Imperial College London.

Anti-AMPKα2 antibody (goat), anti-phospho-Ser16 PLB antibody (rabbit) and anti-phospho-Thr17 PLB antibody (rabbit) were from Santa Cruz Biotechnology Inc.

SAMS peptide was from Cambridge Research Biochemicals.

[γ-^{33}P] ATP, [9,10-^{3}H] palmitate, and [U-^{14}C]-glucose were from N.E.N.

Anti-phospho-Thr172 AMPKα antibody (rabbit), anti-phospho-Ser485/491 AMPKα antibody (rabbit), anti-AMPKα antibody (rabbit), anti-phospho-Ser79 ACC1 antibody (rabbit), anti-ACC antibody (rabbit), anti-phospho-Thr308 PKB antibody (rabbit), anti-phospho-Ser473 PKB antibody (rabbit), anti-PKB antibody (rabbit), HRP-linked anti-rabbit secondary antibody and ECL detection kit (LumiGLO^{TM}Reagent and Peroxide) were from Cell Signalling Technology.

IRDye^{TM}800-conjugated anti-rabbit secondary antibody was from Rockland.

Odyssey blocking buffer was from Licor.

NuPAGE® 4-12% Bis-Tris gels, PVDF membranes, and buffers used for SDS-PAGE and western blotting (NuPAGE® LDS Sample Buffer, Sample Reducing Agent, Antioxidant, MOPS SDS Running Buffer, and Transfer Buffer) were from Invitrogen.

2.2 Laboratory preparations

2.2.1 Palmitate bound to albumin

Sodium palmitate was added to a solution containing 15% (w/v) of bovine serum albumin (BSA) and 0.9% (w/v) NaCl (250mg sodium palmitate was added per 50ml of the solution). The mixture was sonicated in a sonicating water bath until an even mixture was obtained. The mixture was incubated at 50°C for 30 minutes followed by incubation at 4°C overnight. Undissolved sodium palmitate was removed by filtration through Whatman No.1 filter paper at 4°C, followed by centrifugation at 26,000g for 30 minutes at 4°C in a Beckman Sorvall RC5-B centrifuge. The supernatant was collected and the pH was adjusted to 7.4 with NaOH. After determining the concentration of palmitate bound to albumin (Section 2.13), the preparation was aliquoted and stored at -20°C.

2.2.2 AMPK antibody bound to protein G

0.4ml of Protein G-Sepharose was washed twice with Hepes Buffer containing 50mM Hepes pH7.4, 50mM NaF, 5mM $Na_4P_2O_7$, 10% (v/v) glycerol, 1mM EDTA, and 1mM dithiothreitol (DTT). 100µl of either anti-α1 or α2 AMPK antiserum, or pre-immune goat/sheep serum (as controls) was added to 0.8ml of Protein G: Hepes Buffer (1:1, v/v) and mixed overnight at 4°C. Protein G-Sepharose bound to AMPK antibodies/non-immune serum proteins was collected by centrifuging at 5200g for 1min at 4°C, washed twice with 1ml of Hepes Buffer, resuspended in Hepes Buffer (1:1, v/v), and stored at 4°C until used.

2.3 Animals

Animals used were male Sprague Dawley rats weighing 200-350g bred in the Biological Service Unit at University College London. Rats were maintained at 20-22°C with constant access to drinking water and to Rat and Mouse Breeding Diet (Special Diet Services, Witham, Essex, UK) containing 21% protein, 4% fat, 39% starches and sugars. Rats were exposed to 13 hours of light between 0600 and 1900h.

2.4 Heart perfusion

Rats were anesthetised by an intraperitoneal injection of sodium pentobarbitone (300mg/Kg). Hearts were removed and placed in ice-cold Krebs Henseleit Bicarbonate Buffer (KHB) (25mM $NaHCO_3$, 118.5mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, pH7.4) containing 5mM glucose and 1.3mM $CaCl_2$, followed by retrograde perfusion through the aorta, as previously described (Mowbray and Ottaway 1973), with KHB containing 2% (w/v) BSA, 1.3mM $CaCl_2$, 5mM glucose with or without palmitate bound to albumin (Section 2.2.1) as specified. The perfusion medium was constantly gassed with O_2:CO_2 (95%:5%) and maintained at 37°C. The perfusion pressure (preload) was 70±5cm H_2O and the perfusion rate was approximately 15ml/min. All hearts were initially perfused for 5 minutes in non-recirculation mode to remove blood and endogenous hormones. Perfusion was then continued for further 60 minutes with recirculating perfusate, except for the hearts perfused with adrenaline, which were perfused in non-recirculation mode for the entire perfusion period. At the end of perfusion, hearts were excised from the cannula and freeze-clamped using tongs pre-cooled to the temperature of liquid N_2. Frozen hearts were powdered under liquid N_2 and stored in liquid N_2.

2.5 Adult rat cardiac myocyte isolation

The buffers used in the isolation procedure were Buffer A consisting of KHB + 10mM glucose + 1.3mM $CaCl_2$, Buffer B consisting of KHB + 10mM glucose + 0.1mM EGTA, Buffer C consisting of KHB + 10mM glucose + 0 to 5μM added $CaCl_2$, Wash Buffer consisting of KHB + 10mM glucose + 0.2mM $CaCl_2$ + 2% BSA + 0.5mg/ml DNase I, and Resuspension Buffer consisting of KHB + 1.3mM $CaCl_2$ + 2% BSA + 5mM glucose with or without 0.5mM palmitate as indicated. All buffers were prepared with purified H_2O with minimal contamination with Ca^{2+}. Buffers (except for ice-cold Buffer A) were maintained at 37°C and constantly gassed with O_2:CO_2 (95%:5%). Rats were anaesthetised and hearts were removed to ice-cold Buffer A, followed by a brief washout perfusion at 37°C with Buffer A as described above. After perfusion with recirculating Buffer A for 10 minutes, the perfusate was

switched to Buffer B and Ca^{2+} was washed from the heart and perfusion tubing by non-recirculating perfusion with approximately 100ml of Buffer B. Perfusion with Buffer B was continued for a further 5 minutes followed by washing with Buffer C to remove EGTA. Hearts were then perfused with Buffer C containing 0.1% (w/v) BSA, 1mg/ml collagenase (type 2), and 0.4mg/ml hyaluronidase for 20 to 30 minutes until the heart became soft. The exact amount of Ca^{2+} added to Buffer C (<5μM) depended on the batch of digestive enzymes and the level of Ca^{2+} contamination, and was determined empirically. Myocytes were gently dispersed in Buffer C containing 0.1% BSA, 1mg/ml collagenase, 0.4mg/ml hyaluronidase, and 0.5mg/ml DNase I by shaking in a water bath (37°C) (~150 oscillations/min) for 10 minutes with gassing (O_2:CO_2 (95%:5%)). DNase I was included to minimise the loss of myocytes due to clumping of the cells by DNA released from dead cells. The digested heart was filtered through nylon mesh and the cells were allowed to settle under gravity for 5 minutes at 37°C. The supernatant, which contained non-settled dead cells, was removed and the cell pellet was resuspended in Wash Buffer. Myocytes were again allowed to settle under gravity for 3 minutes. The supernatant was removed and the cells were resuspended in fresh Wash Buffer. The Ca^{2+} concentration was then gradually increased every 2 minutes (0.4, 0.6, 0.8, 1, 1.3mM) while the cells were shaken gently at 37°C with gassing (O_2:CO_2 (95%:5%)). Finally, myocytes were allowed to settle under gravity for 5 minutes and the cell pellet was resuspended in Resuspension Buffer (cells:buffer 1:10 (v/v)) as a Cell Stock. Except for measurements of glucose and palmitate oxidation, myocytes were incubated in a total volume of 2ml (0.67ml of Cell Stock + 1.33ml of Resuspension Buffer) in siliconised glass scintillation vials at 37°C with gentle shaking and gassing with O_2:CO_2 (95%:5%). At the end of incubation, cells were collected by centrifugation at 950g at 0°C for 20 seconds. Cells were then quickly washed twice with 0.5ml of ice-cold BSA free buffer (KHB + 1.3mM $CaCl_2$ + 5mM glucose). This step was necessary because BSA was found to interfere with the detection of the AMPKα subunit by western blotting. Cells were finally frozen in liquid N_2 and stored in liquid N_2.

2.6 Measurement of cardiac myocyte viability

The viability was determined using a hemocytometer and trypan blue (0.4%) which stains non-viable cells. Under the light microscope, healthy adult rat cardiac myocytes display elongated (rod-shaped) morphology with visible cross striations. Damaged cells appear short (hypercontracted) or spherical. Viability was determined as the percentage of rod-shaped cells which excluded trypan blue. Cells which excluded trypan blue but were hypercontracted or spherical were not considered viable.

2.7 Measurement of glucose oxidation by myocytes

The incubation medium was palmitate-free Resuspension Buffer gassed with O_2:CO_2 (95%:5%) (Section 2.5) containing 5mM [U-^{14}C] glucose (approximately 20μCi/mmol). Myocytes were incubated at 37°C with gentle shaking in a total volume of 2.5ml (0.5ml Cell Stock + 2ml buffer) in sealed siliconised metabolic flasks fitted with centre wells. At the end of the indicated incubation period, 0.2ml of 60% perchloric acid (PCA) and 0.5ml of 1M benzethonium hydroxide were injected through the stopper to the outer compartment (containing myocytes) and the centre well, respectively. After a further 60 minutes incubation to collect [^{14}C] CO_2, the contents of centre wells were transferred to scintillation vials and 10ml of Ecoscint A was added. The radioactivity was measured by scintillation counting (Section 2.14).

2.8 Measurement of palmitate oxidation by myocytes

Myocytes were incubated as above (Section 2.7) except that the incubation medium contained 5mM glucose and 0.5mM [9,10-^{3}H] palmitate (approximately 18μCi/mmol). At the end of the indicated incubation period, PCA was added to the cells as above. [^{3}H] H_2O was separated from [9,10-^{3}H] palmitate by the method used by Saddik and Lopaschuk (Saddik and Lopaschuk 1991). 0.5ml of incubation medium was mixed with 1.88ml of chloroform:methanol (1:2, v/v). 0.625ml of chloroform and 0.625ml of 2M KCl:2M HCl (v/v) were then added. The mixture was vortexed and centrifuged at 2000g at room temperature. The upper aqueous phase

was collected and 0.3ml of chloroform, 0.3ml of methanol, and 0.27ml of 2M KCl:2M HCl were added. The mixture was vortexed and centrifuged as above. The volume of aqueous phase was measured and a 1ml sample was transferred to a scintillation vial and 10ml Ecoscint A was added for scintillation counting. The efficiency of [^{3}H] H_2O extraction with this method was estimated to be 85-90%.

2.9 AMPK activity assay

Frozen myocytes were sonicated (3 X 7 seconds) in 0.5ml of ice-cold Sonication Buffer containing 50mM Tris/HCl pH 7.5, 1mM EDTA, 1mM EGTA, 50mM NaF, 5mM $Na_4P_2O_7$, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1mM DTT, 1mM phenylmethanesulfonyl fluoride (PMSF), 1mM benzamidine, and 4μg/ml soybean trypsin inhibitor. The cell lysate was centrifuged at 13,000g for 10 minutes at 4°C and 60μl of the supernatant was incubated at 4 °C for 2 hours with either anti-AMPK antibody or pre-immune serum prebound to Protein G-Sepharose (Section 2.2.2). The volumes of antibody/Protein G Sepharose used for immunoprecipitation were 15 and 20μl for α1 and α2 AMPK, respectively. These volumes were chosen because they were found to maximally immunoprecipitate AMPK activity from the supernatant. After binding, the immunoprecipitates were collected by centrifugation at 5,200g for 1 minute at 4°C and washed once with 200μl of Sonication Buffer and twice with AMPK Assay Buffer (40mM Hepes pH7, 80mM NaCl, 0.8mM EDTA, 8% glycerol (v/v), and 800μM DTT) by centrifuging as above. The immunoprecipitates were then incubated at 37°C with gentle shaking in a final volume of 50μl of AMPK Assay Buffer which additionally contained 200μM SAMS peptide (HMRSAMSGLHLVKRR), 200μM [γ-^{33}P] ATP (100-500 dpm/pmol), 5mM $MgCl_2$, and 200μM AMP. The reaction was started by addition of [γ-^{33}P] ATP. The assay measures incorporation of ^{33}P to SAMS peptide (Davies *et al* 1989), which is based on the sequence surrounding the AMPK phosphorylation site (Ser79) of ACC1, with mutation of Ser77 to alanine to prevent phosphorylation by PKA and addition of two arginines to allow recovery of SAMS peptide by ion exchange after the reaction. The reaction was stopped after 30 minutes by spotting the reaction mixture onto squares of P81 Whatman phosphocellulose ion exchange paper which were then immersed in 1% (v/v) orthophosphoric acid. SAMS peptide phosphorylated with ^{33}P was

separated from [γ-^{33}P] ATP by washing the papers twice in 1% orthophosphoric acid followed by two washes in distilled H_2O (10 minutes each). The papers were air-dried and transferred to scintillation vials and 10 ml of Ecoscint A was added before radioactivity was counted by a scintillation counter. Because α1 and α2 antibodies were raised in sheep and goat, respectively, activity immunoprecipitated with sheep/goat pre-immune serum prebound to Protein G-Sepharose was used as a blank to account for non-selective binding of AMPK with serum proteins and/or Protein G-Sepharose and was subtracted from the activity immunoprecipitated with anti-AMPK antibodies. AMPK activity was expressed either as the percentage of AMPK activity of myocytes incubated under control conditions (as indicated in the figures/tables) or as pmol of ATP incorporated to SAMS peptide per mg of the 13,000g supernatant protein per min. Although 200μM AMP was always included in the assay, preliminary experiments confirmed the allosteric activation of immunoprecipitated AMPK by AMP (Figure 2.1). AMPK activity also increased linearly during the assay time (30 minutes) and showed a good linear correlation with the concentration of 13,000g supernatant protein within the range of concentrations typically used (Figure 2.2).

For heart samples, frozen, powdered heart was weighed and homogenised on ice using an Ultra Turrax tissue disintegrator (100mg of tissue/ml) in Homogenisation Buffer containing 50mM Tris/HCl pH7.8, 0.25M mannitol, 1mM EDTA, 1mM EGTA, 50mM NaF, 5mM $Na_4P_2O_7$, 1mM DTT, 1mM PMSF, 1mM benzamidine, and 4μg/ml soybean trypsin inhibitor. The 13,000g supernatant was obtained and AMPK was immunoprecipitated and assayed as above, except that the immunoprecipitate was washed with Homogenisation Buffer instead of Sonication Buffer. AMPK activity of heart samples was expressed as pmol of ATP incorporated to SMAS peptide per minute per mg of the 13,000g supernatant protein.

2.10 Western blotting

Myocytes were lysed as above (Section 2.9) and proteins in 15-25μl of the 13,000g supernatant were separated by sodium dodecylsulphate polyacrylamide gel

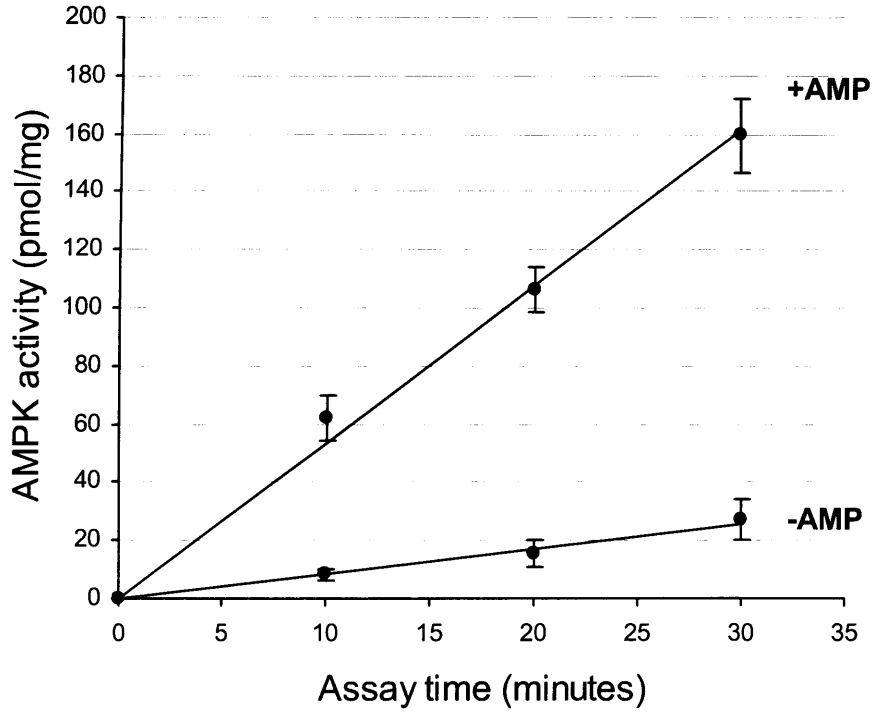


Figure 2.1 Time-course of AMPK activity assay and the allosteric effect of AMP

α2 AMPK immunoprecipitated from cardiac myocyte 13,000g supernatant was assayed for the indicated time in the presence or absence of 200μM AMP, as described in Section 2.9. n=4

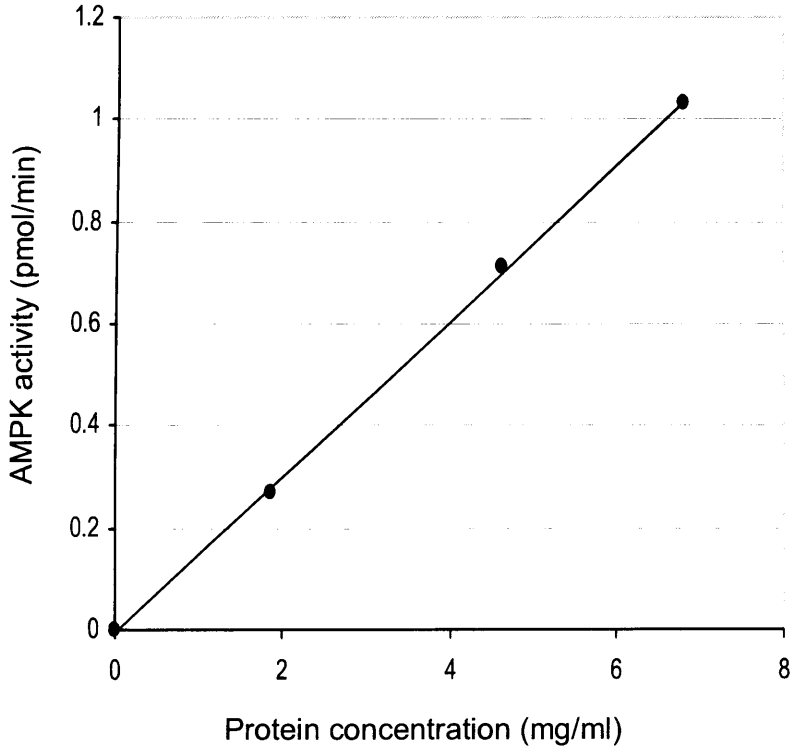


Figure 2.2 The relationship between AMPK activity and protein concentration of the 13,000g supernatant

Samples of 13,000g supernatant with different protein concentrations were prepared from the same myocyte preparation. α2 AMPK was immunoprecipitated from the supernatants and AMPK was assayed in the presence of 200μM AMP as described in Section 2.9. n=1

electrophoresis (SDS-PAGE) under denaturing and reducing conditions on 4-12% Bis-Tris gels, and transferred to polyvinyldene difluoride (PVDF) membranes. Phospho-AMPK Thr172, phospho-AMPK Ser485/491, phospho-PKB Thr308, phospho-PKB Ser473, phospho-ACC Ser79/227, total AMPKα, total PKB, and total ACC were detected by chemiluminescence (Fujifilm LAS-1000 Image Reader) with horseradish peroxidase (HRP)-conjugated secondary antibody and chemiluminescent substrate (luminol). For these measurements, primary and secondary antibodies were diluted (1/1000 dilution) in Tris Buffered Saline (TBS) (20mM Tris/HCl pH7.6, 137mM NaCl) containing 5% (w/v) BSA and 0.1% (v/v) Tween-20. Secondary antibody used for detection of phospho-AMPK Ser485/491 was diluted in the same buffer which additionally contained 0.01% SDS. 5% (w/v) milk powder in TBS + 0.1% Tween-20 was used to block membranes except for membranes probed with anti-phospho-AMPK Ser485/491 antibody, which was blocked with 5% BSA in TBS + 0.1% Tween-20. Band intensities were quantified with Fujifilm Multi Gauge software. After measurements of phospho-AMPK, ACC, or PKB, membranes were stripped by incubating with 62.5mM Tris/HCl buffer, pH 6.7, containing 0.8% (w/v) β-mercaptoethanol and 2% (w/v) SDS at 50°C for 30 minutes, and reprobed with antibodies against total AMPK, ACC, or PKB. Phosphorylation of PLB Ser16 and Thr17 were detected and quantified using secondary antibody conjugated with infrared dye (IRDye^{TM}800) and an Odyssey infrared imaging system. For detection of PLB phosphorylation, membranes were blocked with Odyssey blocking buffer. Anti-phospho-PLB antibodies and secondary antibody were diluted (1/200 (primary antibodies) and 1/2000 (secondary antibody)) in Odyssey blocking buffer + 0.1% Tween-20.

2.11 Measurements of adenine nucleotides and creatine compounds

Adenine nucleotides and creatine compounds were measured by ion-pair reverse phase HPLC as described by Sellevold *et al* (Sellevold *et al* 1986). The column used was a 150 X 4.6mm Nucleosil C18 column with particle size of 5μm and pore size of 100Å. The mobile phase consisted of 215mM KH_2PO_4, 2.3mM tetrabutylammonium hydrogen sulphate (TBAHS), and 3.5% (v/v) acetonitrile. The pH was adjusted to 6.25 with 10M KOH. The mobile phase was filtered through a 0.22μm Millipore

filter and degassed before use. 200µl of ice-cold 0.21M PCA was added to frozen myocytes and the mixture was vortexed. Following centrifugation at 13,000g for 5 minutes at 4°C, perchloric ion in 150µl of the supernatant was precipitated with 1M KOH which was added in sufficient amount to precipitate most of the perchloric ion in the extract whilst maintaining an acidic pH. The precise amount was determined by titration of a sample extract and the usual amount was 20-40µl. Potassium perchlorate precipitates were removed by centrifuging at 13,000g for 2 minutes at 4°C, and 450µl of the mobile phase was added to 50µl of the supernatant. The samples diluted with the mobile phase were filtered through a 0.22µM filter and 50µl was injected onto the column which was pre-equilibriated with the mobile phase. Adenine nucleotides and creatine compounds were eluted isocratically at ambient temperature. The flow rate was 1.0ml/min and each run lasted for 20 minutes. Elution of creatine compounds and adenine nucleotides was detected by changes in absorbance at 206nm using a Waters tunable absorbance detector, and the elution profile was stored and processed using Millennium 2010 software. Peaks for creatine, creatine phosphate, ATP, ADP, and AMP were identified by comparison of the retention times with standards (Figure 2.3). In order to ensure that the standards had a similar amount of residual PCA as the samples, the standards were prepared in 0.21M PCA which was partially neutralised with the same amount of 1M KOH as used in the preparation of the samples, and diluted ten times with the mobile phase before injection onto the column. Amounts of adenine nucleotides and creatine compounds were determined by the comparison of peak areas and peak heights, respectively, with those of the standards. In order to establish whether this method is sensitive enough to accurately measure the small amounts of adenine nucleotides and creatine compounds present in myocyte samples, standards were prepared in the range of concentrations similar to those seen in myocyte samples. Peak areas or peak heights showed a good linear correlation with the amount of adenine nucleotides or creatine compounds (Figures 2.4 and 2.5). Adenine nucleotides and creatine compounds in heart samples were determined as above except that 0.2g of powdered frozen heart was homogenised on ice (1 minute) in 2ml of 0.21M PCA using an Ultra Turrax tissue disintegrator.

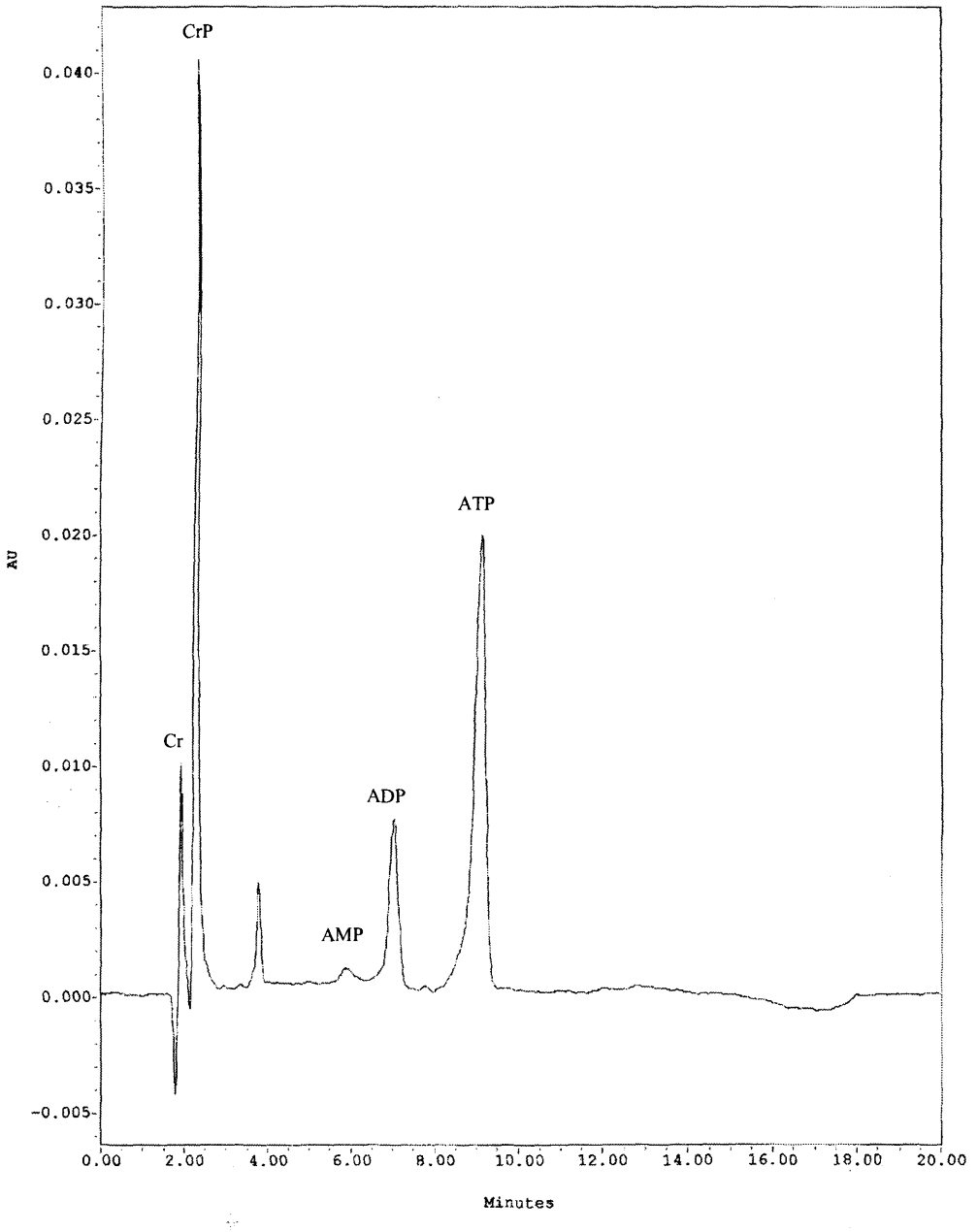


Figure 2.3 A chromatogram of standard solutions of ATP, ADP, AMP, creatine, and creatine phosphate

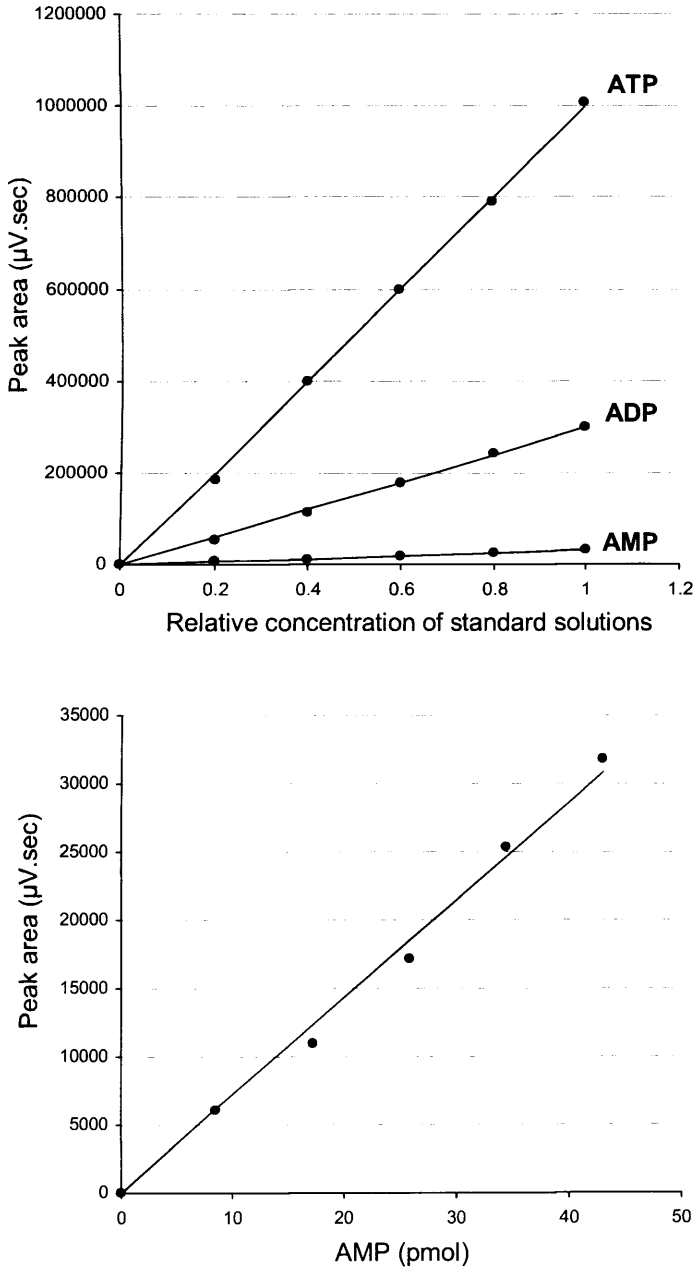


Figure 2.4 The relationship between the amount of ATP, ADP, and AMP with the HPLC peak areas

Standard solutions of ATP, ADP, and AMP were analysed by HPLC as described in Section 2.11. n=1

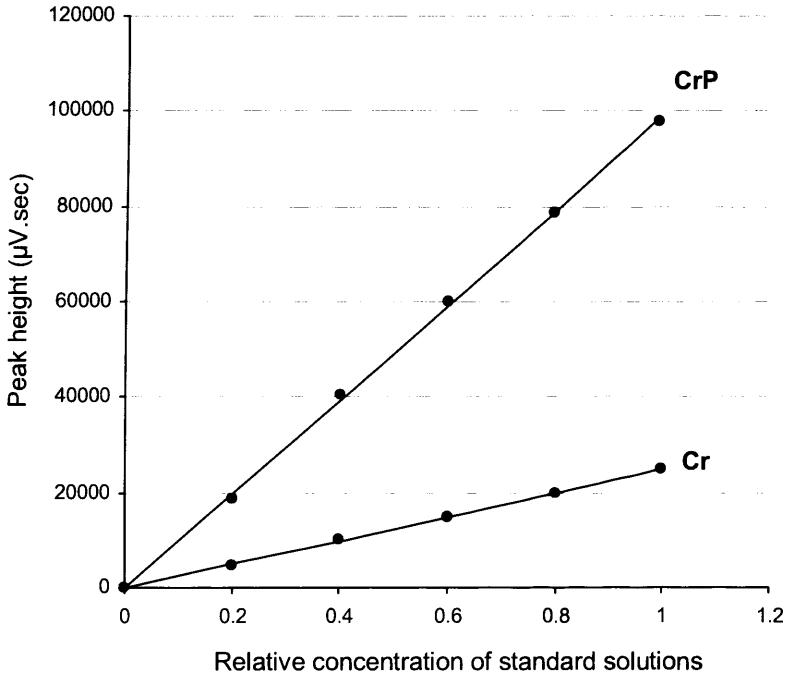


Figure 2.5 The relationship between the amount of creatine and creatine phosphate with the HPLC peak heights

Standard solutions of creatine and creatine phosphate were analysed by HPLC as described in Section 2.11. n=1

2.12 Determination of protein concentration

Protein concentration was determined by a bicinchoninic acid (BCA) method. This method is based on the concentration-dependent reduction of alkaline Cu(II) to Cu(I) by proteins, and the chromogenic reaction of BCA with Cu(I). 10µl of samples were incubated at 37°C for 30 minutes with 200µl of BCA:copper (II) sulphate pentahydrate 4% solution (50:1, v/v) in a 96 well microtitre plate. After incubation, the absorbance at 570nm was determined using a Dynatech MR7000 spectrophotometer. BSA solutions (0.2-1mg/ml) were used as standards.

2.13 Determination of fatty acid concentration

The concentration of free fatty acids was assayed using a Wako NEFA C test kit. This assay is based on the oxidation of fatty acyl-CoA by acyl-CoA oxidase (ACOD) to 2,3-trans-enoyl-CoA. This reaction releases H_2O_2, which, in the presence of peroxidase and 4-aminoantipyrine, allows the conversion of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) to a coloured compound. 10µl of samples were incubated in a 96 well microtitre plate at 37°C for 10 minutes with 67µl of Reagent A (50mM phosphate buffer pH6.9 containing 3mM $MgCl_2$, 0.3U/ml acyl-Coenzyme A synthetase, 3U/ml ascorbate oxidase, 3mg/ml ATP, 0.7mg/ml Coenzyme A, and 0.3mg/ml 4-aminoantipyrine). 133µl of Reagent B (1.2mM MEHA, 6.6U/ml ACOD and 7.5U/ml peroxidase) was then added and samples were incubated for further 10 minutes. The absorbance at 550nm was determined with a Dynatech MR7000 spectrophotometer. Oleate solutions (0.2-1mg/ml) were used as standards.

2.14 β-Scintillation counting

Radioactivity of ^{14}C, ^{3}H, and ^{33}P was measured using a PerkinElmer Tri-Carb 2900TR Liquid Scintillation Analyzer, which is equipped with software which corrected for quenching and allowed direct calculation of activity in disintegrations per minute (dpm).

2.15 Statistical methods

Values are given as means ± S.E.M. Values of n indicate numbers of independent myocyte preparations or perfused hearts. Statistical significance was determined using Students t-test for paired (myocyte data) and unpaired samples (heart data). The following symbols were used throughout in figures/tables to indicate statistical significance: a, $P<0.05$; b, $P<0.025$; c, $P<0.01$; d, $P<0.005$; e, $P<0.0005$; NS, not significant ($P>0.05$). Unless otherwise specified, these symbols indicate statistical significance of values compared to values of cells incubated under control conditions as indicated in legends to figures/tables.

Chapter 3: Results and Discussion

3.1 Cardiac myocyte isolation

Adult rat cardiac myocytes were isolated by an enzymatic perfusion method as detailed in Section 2.5. Key features of the procedure are (1) initial Ca^{2+}-free perfusion in the presence of EGTA, (2) digestion of hearts with a combination of collagenase and hyaluronidase in the presence of low Ca^{2+}, (3) mechanical dispersion of myocytes followed by stepwise reintroduction of physiological Ca^{2+} concentration. As reported by others, careful handling of Ca^{2+} concentration was found to be crucial for successful isolation of cardiac myocytes. Myocytes are usually isolated under low Ca^{2+} to assist their release from intercellular connections and extracellular matrix. One potential problem associated with this is the Ca^{2+} paradox, that is, the loss of viability due to Ca^{2+} overload and hypercontracture when Ca^{2+} is reintroduced to the cells after digestion of hearts under low Ca^{2+} conditions. It has been reported previously that gradual reintroduction of physiological Ca^{2+} concentration lessens the loss of viability caused by the Ca^{2+} paradox (Awan and Saggerson 1993). However, despite this technique, initial attempts to isolate myocytes in the presence of a low concentration of Ca^{2+} in the pre-perfusion (5μM) and enzyme perfusion (50μM) were unsuccessful. This was unlikely to be due to the Ca^{2+} paradox because cells were already non-viable before the reintroduction of a physiological concentration of Ca^{2+}. It was subsequently found that initial viability (before Ca^{2+} introduction) and the yield of Ca^{2+}-tolerant myocytes could be significantly improved if pre-perfusion and enzyme-perfusion were performed in the absence of added Ca^{2+}. There are several explanations for this observation. It is

possible that too much Ca^{2+} during enzyme perfusion may damage myocytes due to excessive enzyme activity because collagenase and possibly other proteases present in commercially available collagenase preparations are activated by Ca^{2+}. Many investigators have also found that a short period of Ca^{2+}-free perfusion is necessary for isolation of viable cells (Tytgat 1994; Mitra and Morad 1985). This may be related to Ca^{2+}-dependent loosening of the extracellular matrix (Mitra and Morad 1985) and intercellular connections (Wittenberg *et al* 1986). Wittenberg *et al* have reported that the concentration of Ca^{2+} has to be reduced to 5-14μM in order to dissociate the intercellular connections (Wittenberg *et al* 1986). A higher concentration of Ca^{2+} increased the proportion of the cells still attached to each other at the intercalated disc, and this was associated with a significant loss of viability, possibly because cells attached firmly to each other are vulnerable to a shearing force of dissociation which forcedly tears apart the cells causing membrane damage. Although hearts in the initial isolation attempts were exposed to Ca^{2+} concentration as low as 5μM, it is possible that the actual concentration of Ca^{2+} was higher than that required for gentle separation of intercellular connections because of contamination from blood, chemicals, and the perfusion apparatus. Although the absence of added Ca^{2+} during the perfusion step could improve viability, over a time, this method showed a considerable variation in the cell viability which ranged from 0-90% even with the same enzyme batches. This was likely to be due to variation in Ca^{2+} contamination. In order to circumvent this problem, EGTA was included during the perfusion step. Addition of EGTA was generally found to improve initial viability and consistency. However, it was noted that in the presence of EGTA, hearts could not be satisfactorily digested due to inhibition of the activity of the dissociation enzymes. The mechanical dispersion of the inadequately digested heart still yielded viable cells but it was observed that these cells were shorter (hypercontracted) than the healthy cells and soon became spherical, possibly due to the damage caused by forced separation of the cells, and that the cells which were not immediately exposed to the buffer during mechanical dispersion became ischemic. Thus, a precise balance of beneficial and detrimental effects of Ca^{2+} was required. Based on these observations, in the final method, hearts were first perfused with Ca^{2+}-free buffer in the presence of EGTA, followed by enzyme perfusion in the presence of a small amount of added Ca^{2+} but in the absence of EGTA. The initial perfusion with EGTA ensured that that heart was exposed to sufficiently low Ca^{2+}.

Omitting EGTA and adding a small amount of Ca^{2+} (<5μM) during the enzyme perfusion ensured adequate digestion of the heart and the gentle release of the myocytes without causing overdigestion. During enzyme perfusion, hyaluronidase was also used in combination with collagenase to facilitate the action of collagenase since heart collagen fibres are coated with a layer of glycoprotein. Addition of Ca^{2+} during enzyme perfusion, however, was not usually found to be required for adequate digestion of hearts, probably due to the presence of Ca^{2+} in the preparations of digestive enzymes. Appropriate amounts of Ca^{2+} were added when underdigestion of the heart was apparent. Consistent isolation of myocytes with reasonably high viability (60-80%) could be achieved with this method.

To assess the metabolic performance of isolated myocytes, glucose and palmitate oxidation by myocytes was determined. Linearity of the rates of glucose and palmitate oxidation for at least 1 hour indicated that the cells are metabolically active and there is no significant loss of viability during this period (Figures 3.1 and 3.2). The values for the rate of oxidation of glucose and palmitate were roughly in the range of values reported by other investigators. Montini *et al* reported glucose (5mM) and palmitate (0.4mM palmitate, 3% BSA) oxidation rates of approximately 1.4 and 0.7nmol/min/mg protein, respectively (Montini *et al* 1981). Burns and Reddy reported glucose (5mM) oxidation rate of approximately 0.7nmol/min/mg protein (Burns and Reddy 1977). The rates of glucose (5mM) and palmitate (0.5mM, 2% BSA) oxidation obtained in the present study were approximately 0.8 and 0.3nmol/min/mg protein, respectively.

3.2 The effect of adrenaline on cardiac AMPK activity

A previous study by Clark *et al* with perfused hearts found that adrenaline could antagonise activation of α2 AMPK by palmitate (Clark *et al* 2004). Because activation of the α1 isoform by palmitate was not affected by adrenaline (Clark *et al* 2004), it appeared likely that the inhibitory effect of adrenaline on α2 AMPK involved a direct modification of the α2 AMPK complex or proteins specifically associated with this isoform. In order to investigate this effect further, it was

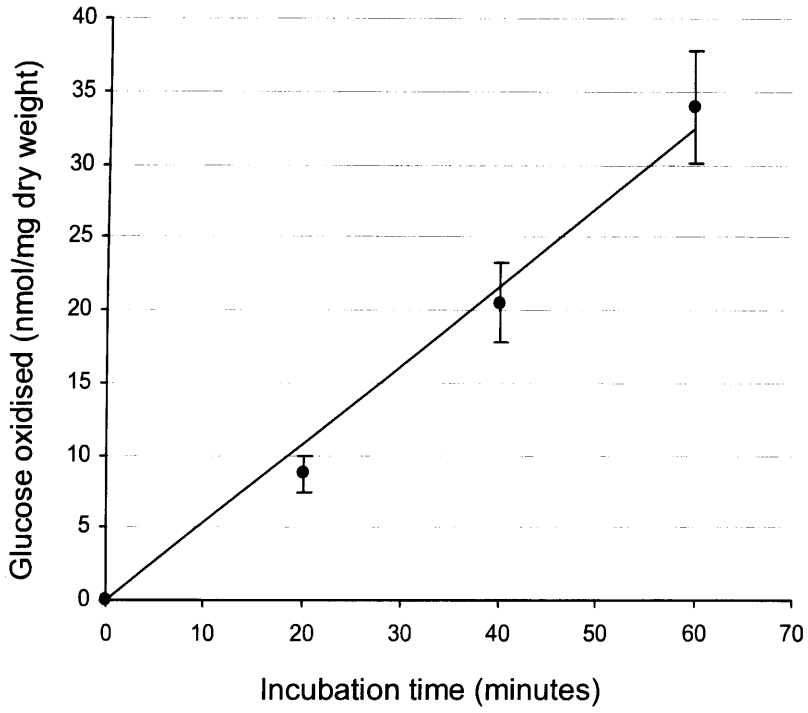


Figure 3.1 Glucose oxidation by cardiac myocytes

Cardiac myocytes were incubated for indicated time in the presence of 5mM [U-^{14}C] glucose. Glucose oxidation was estimated from $^{14}CO_2$ production. n=3

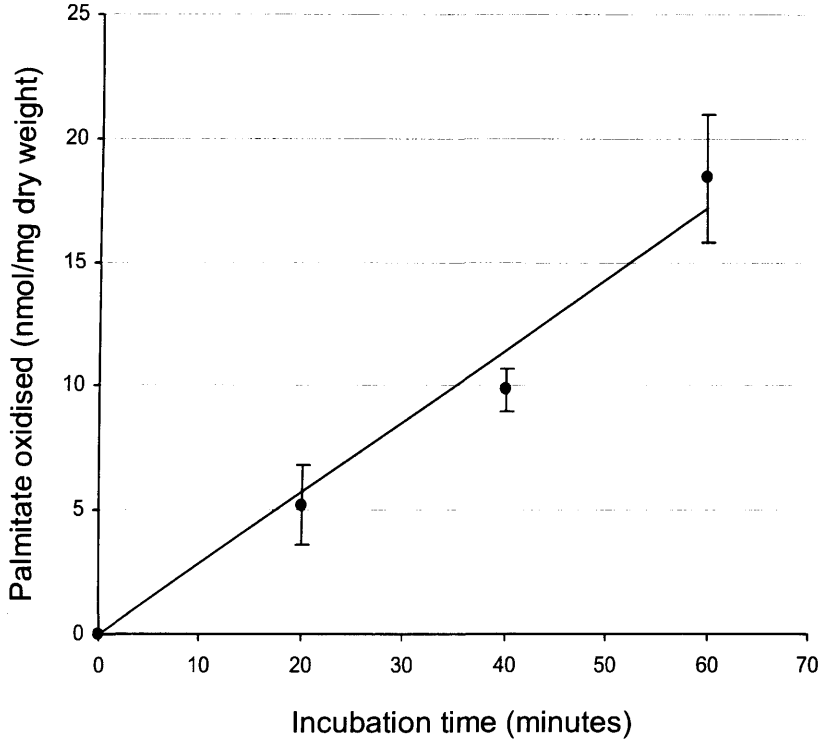


Figure 3.2 Exogenous palmitate oxidation by cardiac myocytes

Cardiac myocytes were incubated for indicated time in the presence of 5mM glucose and 0.5mM [9,11-^{3}H] palmitate (2% BSA). Palmitate oxidation was estimated from ^3H_2O production. n=3

examined whether the effect could be reproduced in cardiac myocytes, an experimental system which is well-suited for dose/time-response analysis and for investigation of signalling pathways. As shown in Figure 3.3, incubation of myocytes with 5mM glucose and 0.5mM palmitate for 1 hour caused a significant 2.5-fold increase in α2 AMPK activity compared to incubation with 5mM glucose alone. The extent of activation of AMPK by 0.5mM palmitate seen in this experiment was comparable to that observed previously in perfused hearts. In the presence of glucose and palmitate, adrenaline (>0.5μM) decreased α2 AMPK activity by approximately 50% (or inhibited the activation of α2 AMPK by palmitate by approximately 70%) (Figure 3.3). The half maximal effect of this inhibitory effect (IC_{50}) was seen at approximately 0.1μM adrenaline. However, in contrast to the observation in perfused hearts that adrenaline did not affect AMPK activity in the absence of palmitate (Clark *et al* 2004), in cardiac myocytes, it was observed that adrenaline decreased AMPK activity to a similar extent (~40-50%) even in the absence of palmitate (Figures 3.4 and 3.16) or in myocytes which did not show a significant activation of AMPK by palmitate (Figure 3.4). Although 2- to 3-fold activation of AMPK by palmitate was consistently observed in the early stage of the present study when the experiment presented in Figure 3.3 was carried out, this marked activation of AMPK by palmitate could not be reproduced in the later experiments. The reason for this is unknown. Because the activation of AMPK by palmitate was found to be a rather slow process in the previous study with perfused hearts (the activation was apparent only after 1 hour of perfusion), it was examined whether extending the time of incubation from 1 hour to 2 hours could enhance the activation of AMPK by palmitate in the later myocyte preparations (Figure 3.4). Linearity of palmitate oxidation indicated myocytes were metabolising palmitate and that there was no marked loss of viability during this extended incubation period. However, no significant effect of palmitate on AMPK activity was observed even after 2 hours. Nonetheless, adrenaline still significantly decreased AMPK activity by ~50% in these cells in the presence of palmitate. These results suggest a direct inhibitory effect of adrenaline on cardiac AMPK activity, at least in isolated myocytes. The effect of adrenaline in cardiac myocytes was not dependent on the presence of glucose. Under conditions where palmitate had little or no effect on AMPK, 2μM adrenaline still decreased α2 AMPK activity of myocytes incubated with 0.5mM

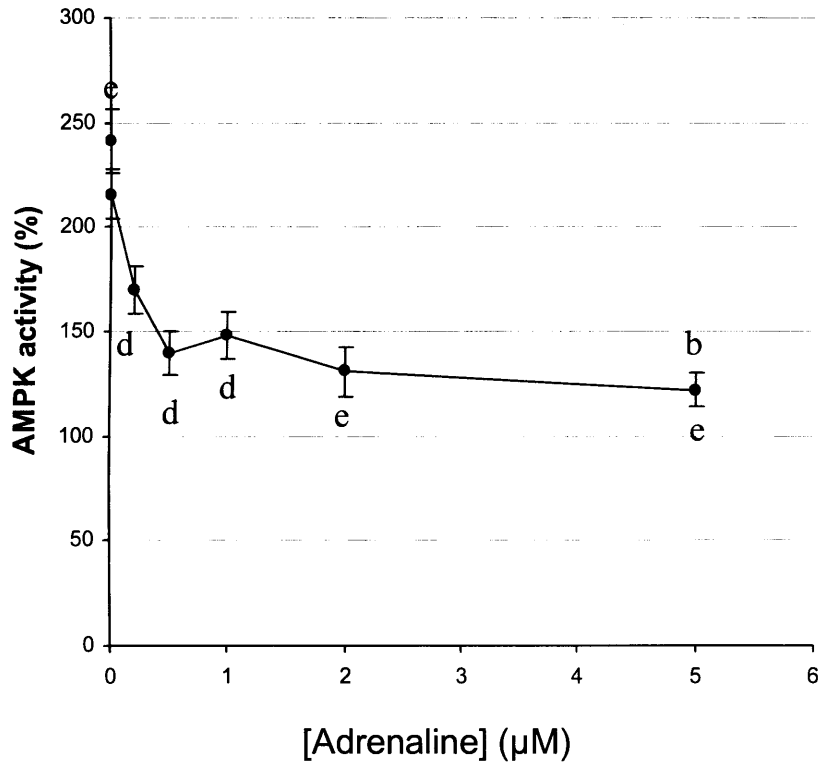


Figure 3.3 The effect of palmitate on α2 AMPK activity and the dose-response curve of the inhibition of AMPK by adrenaline in cardiac myocytes

Cardiac myocytes were incubated for 1 hour with 5mM glucose alone or with 5mM glucose + 0.5mM palmitate in the absence or presence of the indicated concentrations of adrenaline. α2 AMPK activity was assayed in the presence of 200μM AMP. The results are expressed as percentage of AMPK activity of cells incubated with 5mM glucose alone (without adrenaline). The letters above the data points indicate statistical significance compared to AMPK activity of cells incubated with 5mM glucose alone (without adrenaline). The letters below the data points indicate statistical significance compared to AMPK activity of cells incubated with 5mM glucose + 0.5mM palmitate in the absence of adrenaline. n=7-16

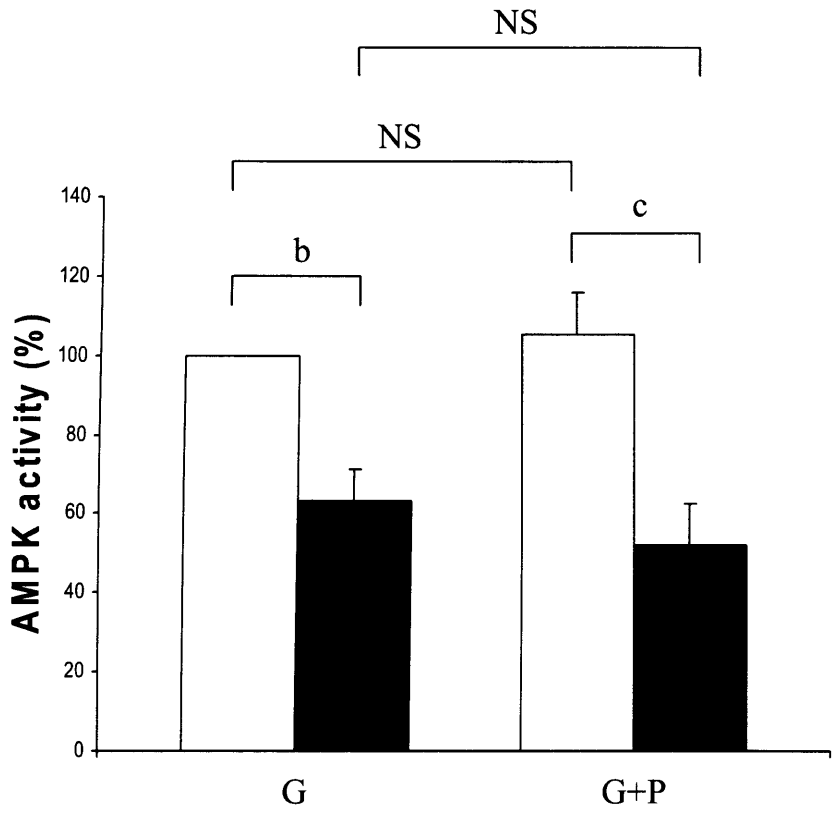


Figure 3.4 The presence of palmitate is not essential for the effect of adrenaline to decrease AMPK activity

Cardiac myocytes were incubated for 2 hour with 5mM glucose alone (G) or 5mM glucose + 0.5mM palmitate (G+P) in the absence (open bar) or presence (filled bar) of 2μM adrenaline. α2AMPK activity was assayed in the presence of 200μM AMP. The results are expressed as percentage of AMPK activity of cells incubated with 5mM glucose alone in the absence of adrenaline. n=3

palmitate alone by 41±4% ($P<0.005$, n=4) after 1 hour. In the same myocytes preparations adrenaline decreased α2 AMPK activity of cells incubated with 5mM glucose and 0.5mM palmitate by 37±6% ($P<0.01$, n=4). In the rest of the experiments presented here, myocytes were normally incubated in the presence of both 5mM glucose and 0.5mM palmitate to ensure adequate fuelling of the cells. However, because these experiments were performed under conditions where palmitate had little or no effect on AMPK activity, these experiments effectively investigated the direct inhibitory effect[1] of adrenaline on AMPK activity in cardiac myocytes. At present, the possibility cannot be ruled out that this direct inhibitory effect is mechanistically distinct from the effect of adrenaline to block activation of AMPK by palmitate observed previously in perfused hearts and in the results presented in Figure 3.3. In contrast to the inhibitory effect of adrenaline reported previously in perfused hearts, which was restricted to α2 AMPK, the direct effect of adrenaline on AMPK in cardiac myocytes was seen for both α1 and α2 isoforms, although the effect was greater for α2 AMPK (Figure 3.5).

In order to re-investigate the possibility of a direct inhibitory effect of adrenaline on AMPK activity in perfused hearts and to check if the loss of the effect of palmitate to activate AMPK was due to the myocyte isolation procedure, hearts were perfused for 1 hour with 5mM glucose or 5mM glucose + 0.5mM palmitate in the presence or absence of 2μM adrenaline (Figure 3.6). Palmitate did not activate α2 AMPK in perfused hearts, indicating the lack of the effect of palmitate in myocytes was not due to cell damage caused during the isolation procedure. In contrast to the observation in myocytes, adrenaline did not affect α2 AMPK activity in hearts perfused with glucose alone or glucose and palmitate, the observation consistent with the lack of a direct effect of adrenaline in perfused hearts reported previously by Clark *et al.* Therefore, the possibility was considered that the apparently direct inhibitory effect of adrenaline seen in myocytes in the present study was an experimental artefact. While screening for different conditions to activate AMPK in cardiac myocytes, it was found that incubation of cells with 0.5mM H_2O_2 for 1 hour increased α1 AMPK activity to 227.5±40.4% relative to control ($P<0.05$) (n=4), but apparently decreased

[1] The expression 'direct inhibitory effect' is used here simply to distinguish an inhibitory effect of adrenaline on AMPK activity under basal conditions from that seen when AMPK is activated by palmitate. It does not necessarily mean that the effect of adrenaline on AMPK under basal conditions is mechanistically direct.

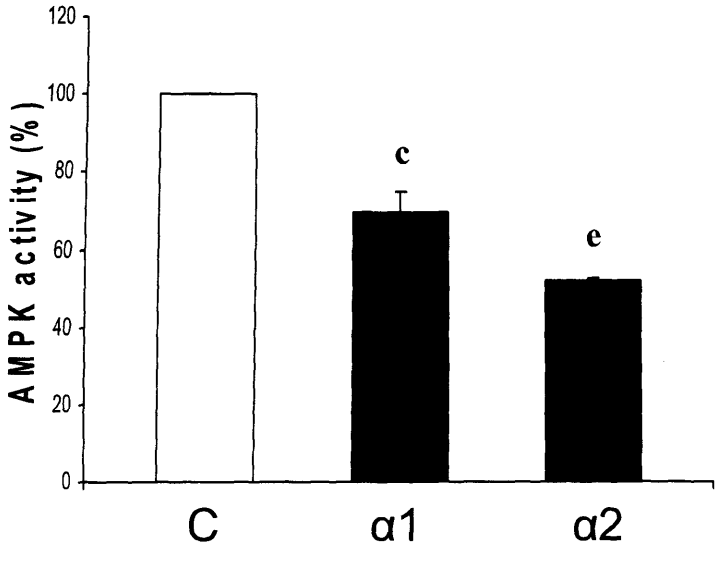


Figure 3.5 The effect of adrenaline on α1 and α2 AMPK activity

Cardiac myocytes were incubated for 1 hour with 5mM glucose and 0.5mM palmitate in the absence (C) or presence of 2μM adrenaline (filled bars). Following immunoprecipitation using anti-α1 or α2 AMPK antibody, AMPK activity was assayed in the presence of 200μM AMP. The results are expressed as percentage of α1 or α2 AMPK activity of cells incubated in the absence of adrenaline. n=4

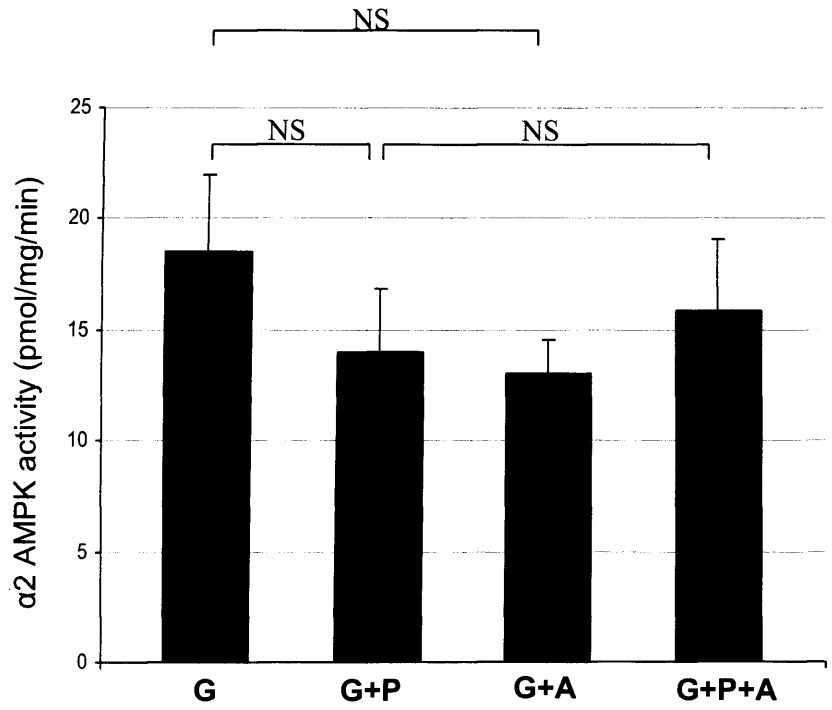


Figure 3.6 The effect of palmitate and adrenaline on α2 AMPK activity in perfused hearts

Hearts were perfused for 1 hour with 5mM glucose alone (G), 5mM glucose + 0.5mM palmitate (G+P), 5mM glucose + 2μM adrenaline (G+A), or 5mM glucose + 0.5mM palmitate + 2μM adrenaline (G+P+A). AMPK activity was assayed in the presence of 200μM AMP. n=4-6

α2 AMPK activity to 27.0±7.0% (P<0.005) (n=6). It has been previously reported in skeletal muscle that H_2O_2 activates α1 AMPK but it does not affect α2 AMPK activity (Toyoda *et al* 2004). The apparent inhibition of α2 AMPK was due to a reduction in cell viability to approximately 26% (n=2) of the untreated cells and loss of AMPK protein which was decreased to 40.0±7.2% (P<0.005) (n=4). Unphysiologically high concentrations of adrenaline as used in this study (μM) have been reported to damage the heart as evidenced by release of lactate dehydrogenase after 10 minutes of perfusion with adrenaline (Horak and Opie 1983). Prolonged adrenergic stimulation is also known to cause apoptosis in cardiac myocytes (Zhu *et al* 2003). However, the decrease in AMPK activity by adrenaline seen in the present study was unlikely to be due to such toxic effects of adrenaline because adrenaline treatment did not significantly affect the viability of myocytes (initial viability: 71±5%; viability after 1 hour incubation: 65±3%; viability after 1 hour incubation with 2μM adrenaline: 67±2%; n=6). Moreover, the decrease in AMPK activity by adrenaline was accompanied by a corresponding decrease in Thr172 phosphorylation in the AMPK α subunit (Figure 3.7) without affecting the α subunit protein amount (100±12.5% relative to untreated; n=4). Adrenaline also caused a significant decrease in Ser227 (Ser79 in ACC1) phosphorylation of ACC2 (the predominant cardiac isoform) (Figure 3.8), a well-established downstream target of AMPK, again without affecting the total ACC amount (107.0±14.9% relative to untreated, n=4). Therefore, it appears that adrenaline has a genuine direct inhibitory effect on AMPK in cardiac myocytes.

Figure 3.9 shows a time-course of the effect of adrenaline to decrease α2 AMPK activity in cardiac myocytes. This and all subsequent experiments focussed on the α2 isoform because of the greater effect of adrenaline on this isoform (Figure 3.5) and because α2 AMPK is the predominant isoform in the heart (Cheung *et al* 2000). The effect of adrenaline was rapid with the half-maximal effect seen at approximately 4 minutes, and the effect was sustained after 1 hour (Figure 3.9) or 2 hours of incubation (Figure 3.4).

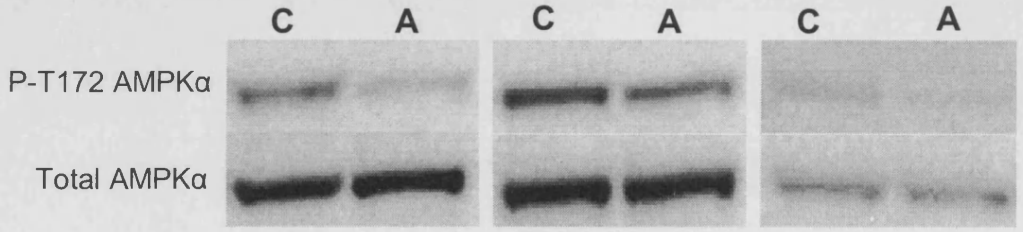
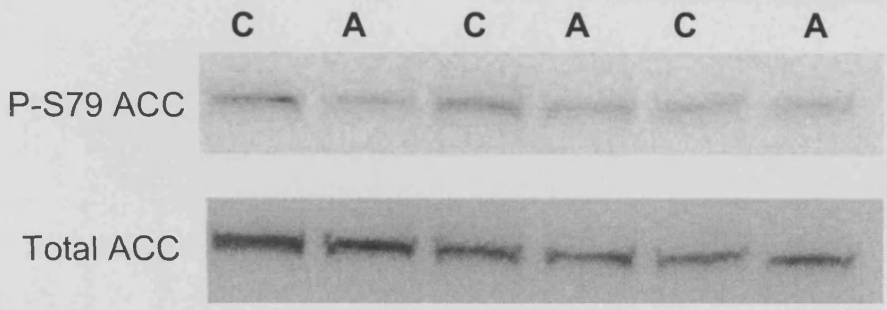


Figure 3.7 The effect of adrenaline on AMPK Thr172 phosphorylation in cardiac myocytes.

Cardiac myocytes were incubated for 1 hour with 5mM glucose and 0.5mM palmitate in the absence (C) or presence of 2μM adrenaline (A). The figure shows the results from three myocyte preparations. Band quantification is given in Figure 3.19.

(a)



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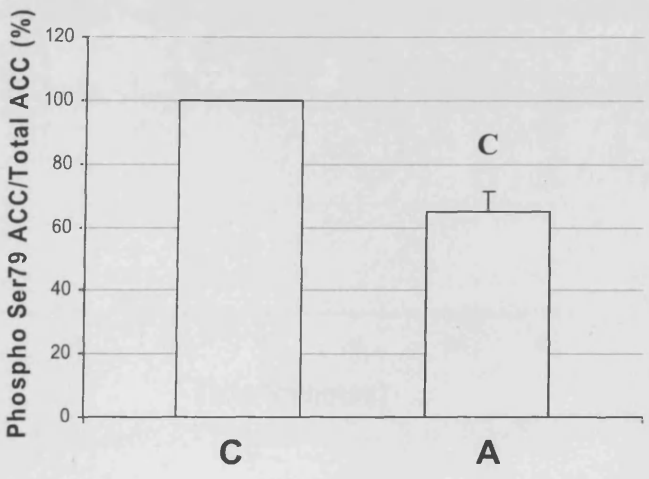


Figure 3.8 The effect of adrenaline on ACC Ser79 (Ser227) phosphorylation in cardiac myocytes.

(a) Cardiac myocytes were incubated for 1 hour with 5mM glucose and 0.5mM palmitate in the absence (C) or presence of 2μM adrenaline (A). ACC phosphorylation was detected using an anti-phospho-Ser79 ACC1 antibody, which also reacts with the equivalent residue (Ser227) in ACC2 (predominant cardiac isoform). The figure shows the results from three myocyte preparations. (b) Quantification of band intensity, expressed as percentage of phospho-ACC/total ACC of cells incubated without adrenaline, is shown. n=4

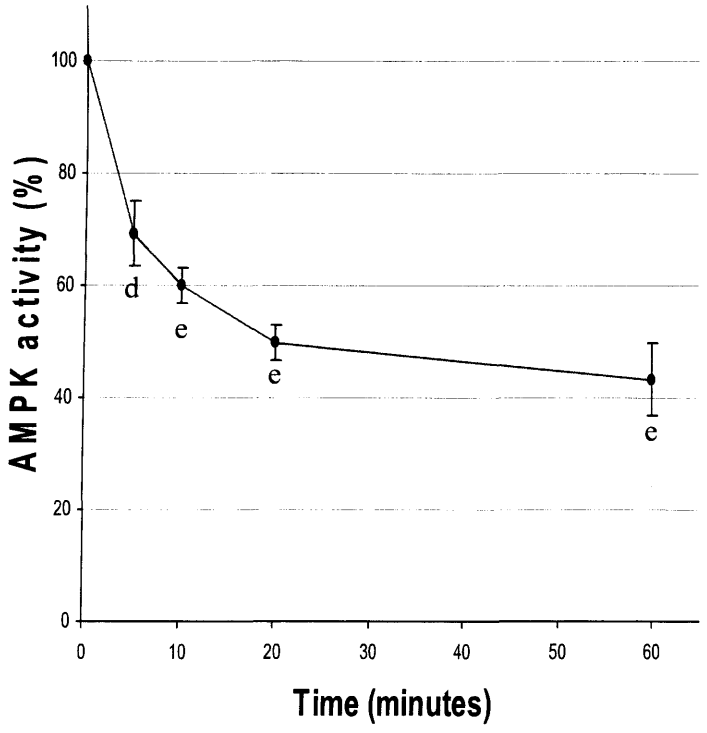


Figure 3.9 Time-course of inhibition of AMPK by adrenaline

Cardiac myocytes were incubated for indicated time with 5mM glucose and 0.5mM palmitate in the absence or presence of 2μM adrenaline. α2 AMPK activity was assayed in the presence of 200μM AMP. The results are expressed as percentage of AMPK activity of cells incubated in the absence of adrenaline. n=5

3.3 The effect of adrenaline is independent of changes in total cellular high energy phosphates

Adrenergic stimulation increases cardiac energy production to meet an increased demand for energy which accompanies an increased contractile activity. Because cardiac myocytes are quiescent, it is conceivable that adrenaline stimulation in the cardiac myocytes increases the energy production and cellular high energy phosphates without significantly increasing the energy utilisation and consequently decreases AMPK activity. This may account for the inhibition of AMPK by adrenaline in myocytes but not in perfused hearts. In order to test this possibility, the total cellular high energy phosphate contents were analysed by HPLC after incubating the cells in the presence or absence of 2μM adrenaline for 20 minutes. No significant difference in the total amount of AMP, ADP, ATP, or the AMP/ATP ratio was observed despite a significant inhibition of the AMPK activity by adrenaline (Table 3.1). The direct regulation of AMPK by Cr and CrP is questioned by a recent study (Taylor *et al* 2005). Nevertheless, the Cr/CrP ratio is a more sensitive indicator of cellular energy state than the AMP/ATP ratio (Neumann *et al* 2002). Adrenaline did not affect the cellular contents of Cr, CrP, or the Cr/CrP ratio. The AMP/ATP ratio was also not affected by adrenaline after 1 hour incubation despite a decrease in AMPK activity to 61.1±4.2% (n=8) ($P<0.0005$). The AMP/ATP ratios of control and adrenaline-treated cells after 1 hour were 0.045±0.011 (n=8) and 0.040±0.009 (n=8), respectively. There was no significant difference between the AMP/ATP ratios between 20 and 60 minutes. Therefore, it appears that the effect of adrenaline to inhibit AMPK in myocytes is not due to increased energy production, and there may be a novel pathway linking the adrenergic receptors to the AMPK system in cardiac myocytes.

3.4 The effect of adrenaline is mediated by both α_1 and β adrenoreceptors

The effect of 2μM adrenaline to inhibit AMPK was partially abolished in the presence of either 2μM propranolol (a selective β blocker) or 2μM phentolamine (a selective α_1 blocker), while the effect was essentially abolished when both blockers

	Control (20 min)	+adrenaline (20 min)
Cr (nmol/mg protein)	9.3 ± 1.3	10.0 ± 0.9^{NS}
CrP (nmol/mg protein)	13.6 ± 1.2	13.1 ± 1.8^{NS}
AMP (nmol/mg protein)	0.398 ± 0.114	0.372 ± 0.058^{NS}
ADP (nmol/mg protein)	3.55 ± 0.41	3.31 ± 0.49^{NS}
ATP (nmol/mg protein)	10.3 ± 0.9	10.3 ± 1.0^{NS}
AMP+ADP+ATP (nmol/mg protein)	14.28 ± 1.25	14.03 ± 1.43^{NS}
Cr/CrP	0.69 ± 0.09	0.79 ± 0.07^{NS}
AMP/ATP	0.039 ± 0.011	0.036 ± 0.005^{NS}
AMPK activity (%)	100	72.5 ± 1.8^{e}

Table 3.1 The effect of adrenaline on adenine nucleotides and creatine compounds

Cardiac myocytes were incubated for 20 minutes with 5mM glucose and 0.5mM palmitate in the absence or presence of 2μM adrenaline. Adenine nucleotides and creatine compounds were measured by HPLC following PCA extraction. The effect of adrenaline on AMPK activity in the same myocytes preparations was also determined in parallel. n=4

were present (Figure 3.10). The effect of adrenaline could be completely mimicked by the β agonist isoproterenol (IC_{50}~40nM) (Figure 3.11). The α_1 agonist phenylephrine did not significantly inhibit AMPK at 2μM, but higher concentrations (10 or 100μM) of phenylephrine completely mimicked the effect of adrenaline (IC_{50}~4μM) (Figure 3.12). Both isoproterenol (2μM) and phenylephrine (100μM) also decreased AMPK Thr172 phosphorylation. Because a high concentration of phenylephrine (100μM) is known to cause cross-stimulation of β receptors (Valks *et al* 2002), cells were incubated with phenylephrine in combination with 2μM propranolol in these experiments. The lack of cross-stimulation of β receptors by phenylephrine was supported by a lack of detectable phosphorylation of PLB at Ser16 (the site phosphorylated exclusively by PKA (Wegener *et al* 1989)) in cells treated with 2, 10, or 100μM phenylephrine (Figure 3.13). As expected, phosphorylation of PLB Ser16 was increased by both adrenaline and isoproterenol (Kuschel *et al* 1999). As a precaution, isoproterenol was given to the cells in combination with 2μM phentolamine. Although the possibility of the cross-stimulation of the α_1 receptor by isoproterenol cannot be completely ruled out in the present study, it is unlikely that the effect of isoproterenol on AMPK was mediated by the α_1 receptor because it was seen with low concentrations of the agonist (0.5μM) despite the presence of 2μM phentolamine. These results indicate that the effect of adrenaline is mediated by both α_1 and β receptors. Several signalling components such as PI3K, Ca^{2+} signalling, and MAPKs are known to be activated by both adrenergic receptors, and it is possible that the effect of adrenaline involves one of these signalling components common to both receptors. However, these results do not rule out the possibility of the two distinct mechanisms to inhibit AMPK by α_1 and β receptors.

3.5 The effect of adrenaline is not mediated through the PI3K/PKB pathway

In perfused hearts, insulin has been shown to decrease basal AMPK activity as well as the activation of AMPK by ischemia by an incompletely understood mechanism (Beauloye *et al* 2001). The effect of insulin has been reported to be dependent on

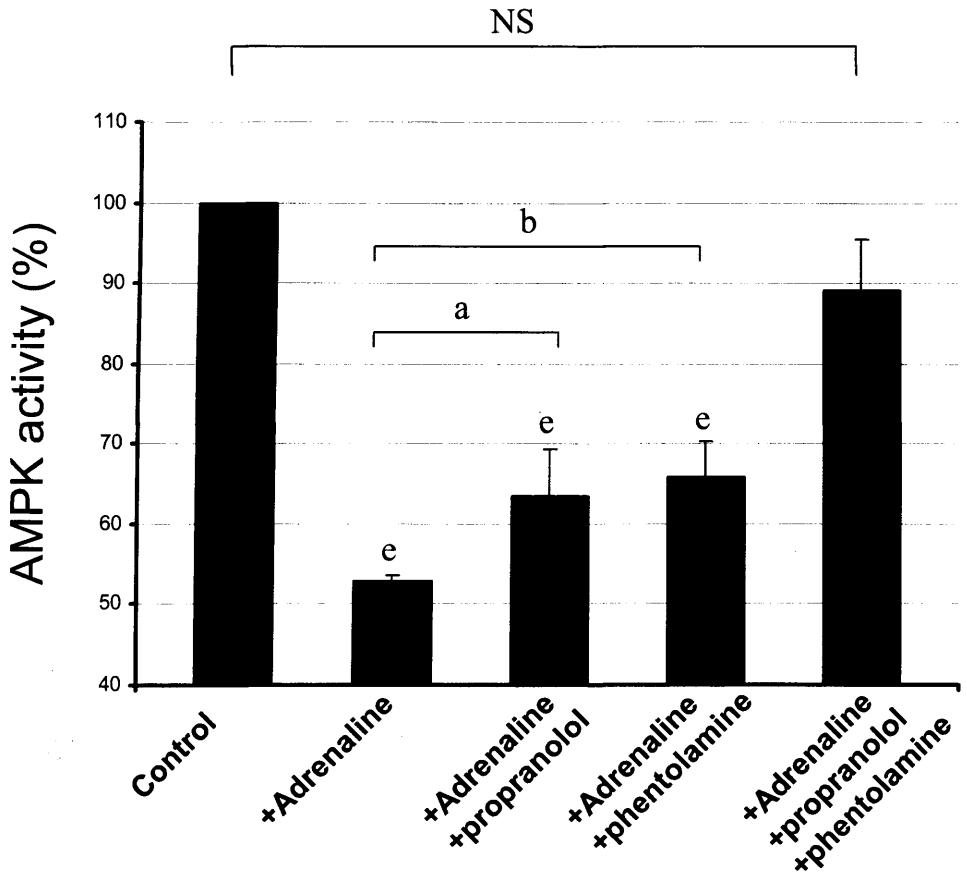
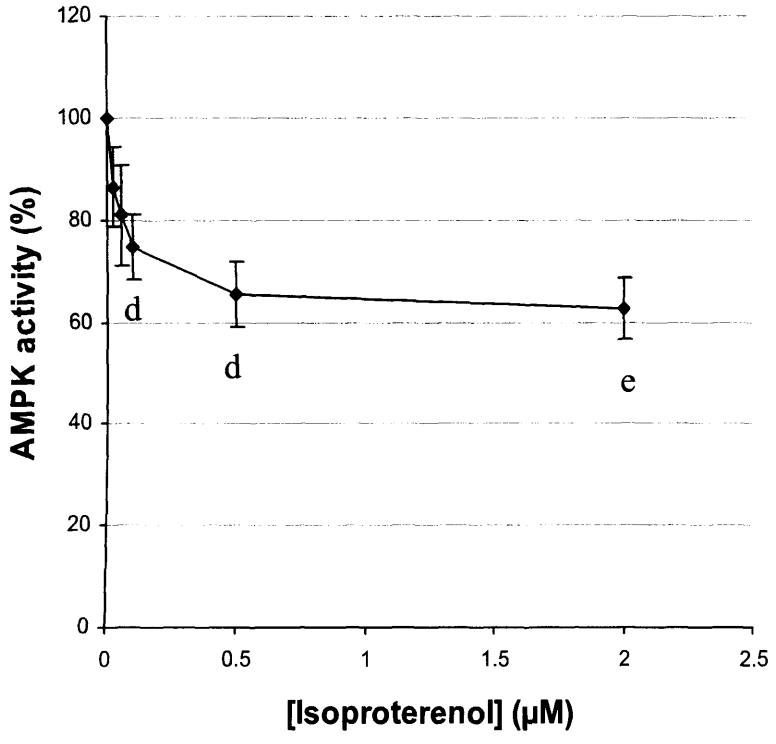


Figure 3.10 The effect of α_1 and β adrenergic antagonists on the effect of adrenaline on AMPK

Cardiac myocytes were incubated for 1 hour with 5mM glucose and 0.5mM palmitate in the absence (control) or presence of 2μM adrenaline and adrenergic antagonists (2μM propranolol and 2μM phentolamine) as indicated. α2 AMPK activity was assayed in the presence of 200μM AMP. The results are expressed as percentage of AMPK activity of control cells. The letters directly above the bars indicate statistical significance compared to control. n=8

(a)



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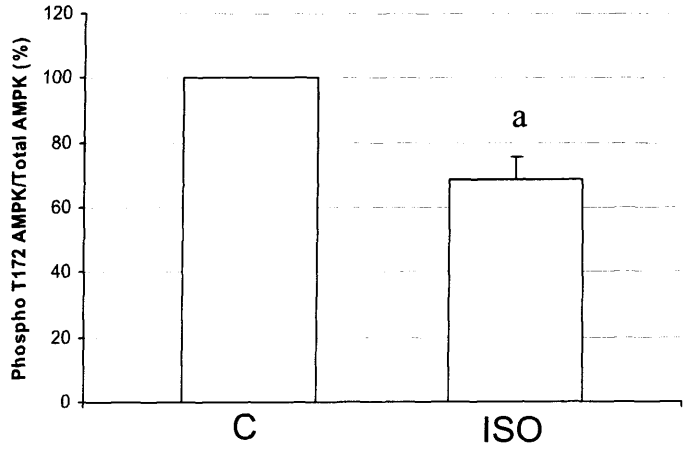
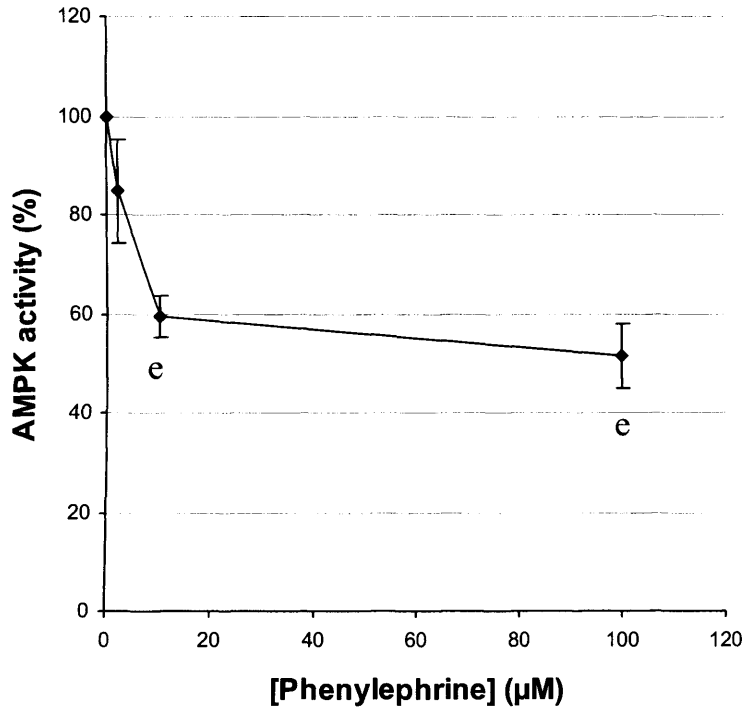


Figure 3.11 The effect of isoproterenol on AMPK activity and Thr172 phosphorylation

(a) Cardiac myocytes were incubated with 5mM glucose and 0.5mM palmitate for 1 hour in the absence (control) or presence of the indicated concentrations of isoproterenol. Isoproterenol was given to the cells in combination with 2μM phentolamine. α2 AMPK activity was assayed in the presence of 200μM AMP. The results are expressed as percentage of AMPK activity of control cells. n=4-10 (b) shows band intensity of phospho-Thr172 from cells treated with 2μM isoproterenol (ISO) corrected for total AMPKα amount and expressed as percentage of the value of control cells (C). n=3

(a)



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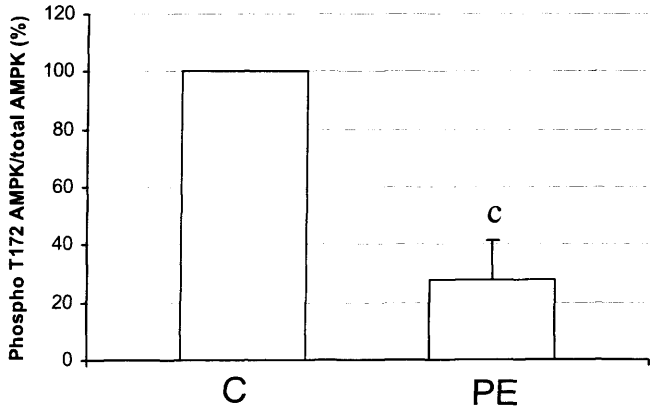


Figure 3.12 The effect of phenylephrine on AMPK activity and Thr172 phosphorylation

(a) Cardiac myocytes were incubated with 5mM glucose and 0.5mM palmitate for 1 hour in the absence (control) or presence of the indicated concentrations of phenylephrine. Phenylephrine was given to the cells in combination with 2μM propranolol. α2 AMPK activity was assayed in the presence of 200μM AMP. The results are expressed as percentage of AMPK activity of control cells. n=6 (b) shows band intensity of phospho-Thr172 from cells treated with 100μM phenylephrine (PE) corrected for total AMPKα amount and expressed as percentage of the value of control cells (C). n=4

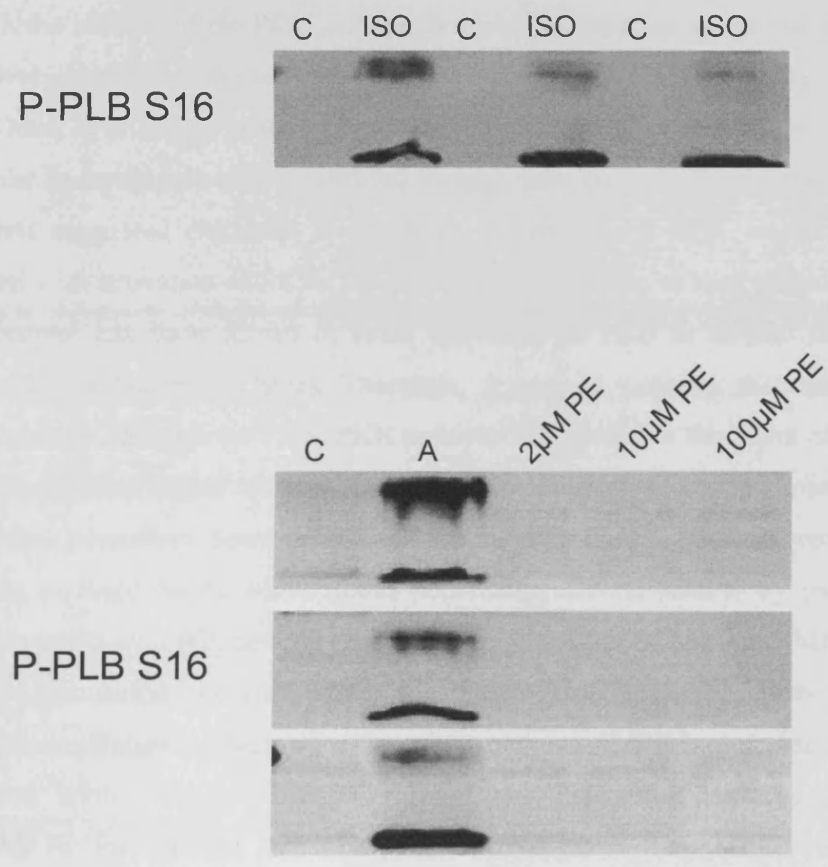


Figure 3.13 The effect of adrenergic agonists on PLB Ser16 phosphorylation

Phosphorylation of PLB Ser16 was examined after incubating cardiac myocytes with 5mM glucose and 0.5mM palmitate for 1 hour in the absence (C) or presence of 2μM adrenaline (A), 2μM isoproterenol + 2μM phentolamine (ISO), or the indicated concentrations of PE + 2μM propranolol (PE). The figures show the results from three myocyte preparations.

PI3K activity, but independent of changes in the AMP/ATP or Cr/CrP ratios (Beauloye *et al* 2001). Kovacic *et al* have shown that the effect of insulin to inhibit AMPK is mimicked by over-expressing constitutively active PKB, suggesting an involvement of PI3K-PKB pathway (Kovacic *et al* 2003). PI3K has been shown to be downstream of both α_1 and β adrenergic receptors in the heart (Section 1.5.5). Although the identity of the PI3K isoform activated by these receptors has not been conclusively elucidated, the activation of the class IB PI3K (PI3Kγ) through the $G_{\beta\gamma}$ dimer (Oudit *et al* 2004), as well as the possibility of the activation of the class IA PI3Ks (the isoforms activated by insulin) through increased tyrosine kinase activity have been suggested (Morisco *et al* 2000). Activation of PI3K is not always associated with activation of PKB (Till *et al* 2000). However, at least stimulation of the β receptor has been shown to cause activation of PKB in cardiac myocytes through PI3K (Zhu *et al* 2001). Therefore, it seemed possible that adrenaline inhibited AMPK through the PI3K-PKB pathway. The fact that the effect of insulin is seen in perfused hearts whereas that of adrenaline is not does not necessarily exclude this possibility because unlike insulin, adrenaline stimulates contractile activity in perfused hearts which could potentially activate AMPK by increasing AMP. Consistent with this, several studies have reported an increase in AMP during adrenergic stimulation in perfused or *in vivo* hearts (Goodwin *et al* 1998b; Ingwall 2002). The simultaneous effects of adrenaline to activate AMPK by increased energy expenditure while inhibiting AMPK through the PI3K-PKB pathway may be responsible for the apparent lack of significant changes in the AMPK activity by adrenaline in perfused hearts. The possibility of an involvement of PI3K in the effect of adrenaline was investigated using wortmannin, an inhibitor of both class IA and IB PI3Ks (Figure 3.14). While wortmannin (3μM) abolished the small significant decrease in AMPK activity by insulin and slightly increased the basal activity, possibly due to removal of some tonic inhibition by PI3K, it did not affect the effect of adrenaline, indicating that PI3K is not involved in the effect of adrenaline. Moreover, in contrast to the observations by other investigators, no increase in the phosphorylation of PKB at Thr308 or Ser473 was detected after 1 hour of incubation with adrenaline (Figure 3.15), suggesting that the PI3K-PKB pathway is not relevant to the effect of adrenaline. As expected, insulin increased phosphorylation of PKB at both Thr308 and Ser473. However, these results do not rule out the involvement of a

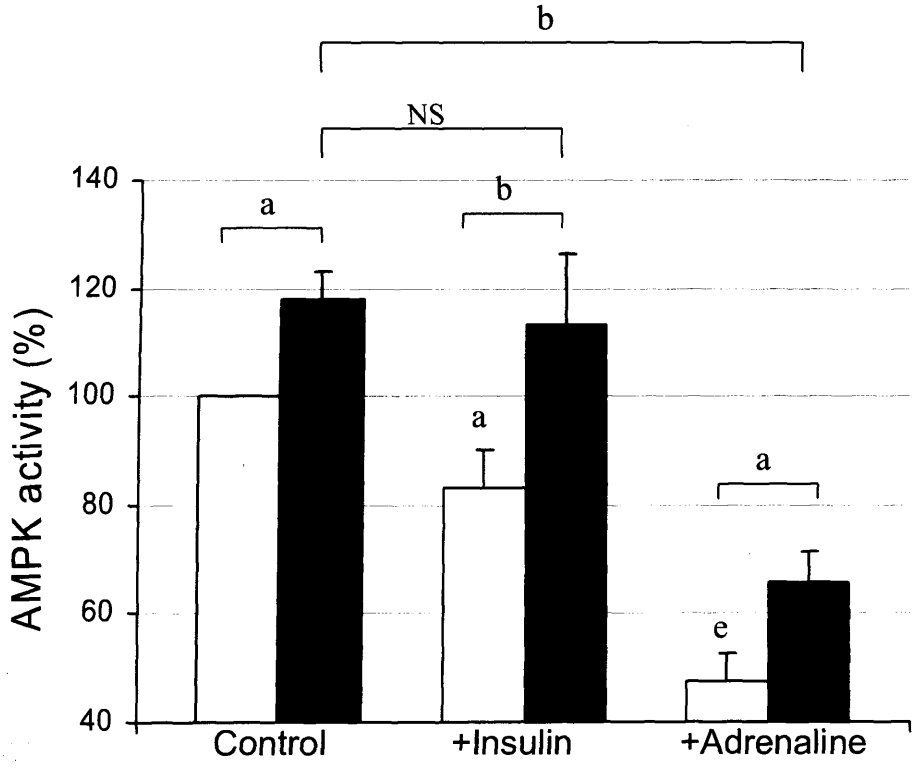


Figure 3.14 The effect of wortmannin on the effect of adrenaline on AMPK

Cardiac myocytes were incubated for 1 hour with 5mM glucose and 0.5mM palmitate alone (control) or with insulin (10nM) or adrenaline (2μM), in the presence (filled bar) or absence (open bar) of 3μM wortmannin. α2 AMPK activity was assayed in the presence of 200μM AMP. The results are expressed as percentage of AMPK activity of cells incubated without insulin, adrenaline or wortmannin. The letters direct above the bars indicate statistical significance compared to control without adrenaline. n=3-5

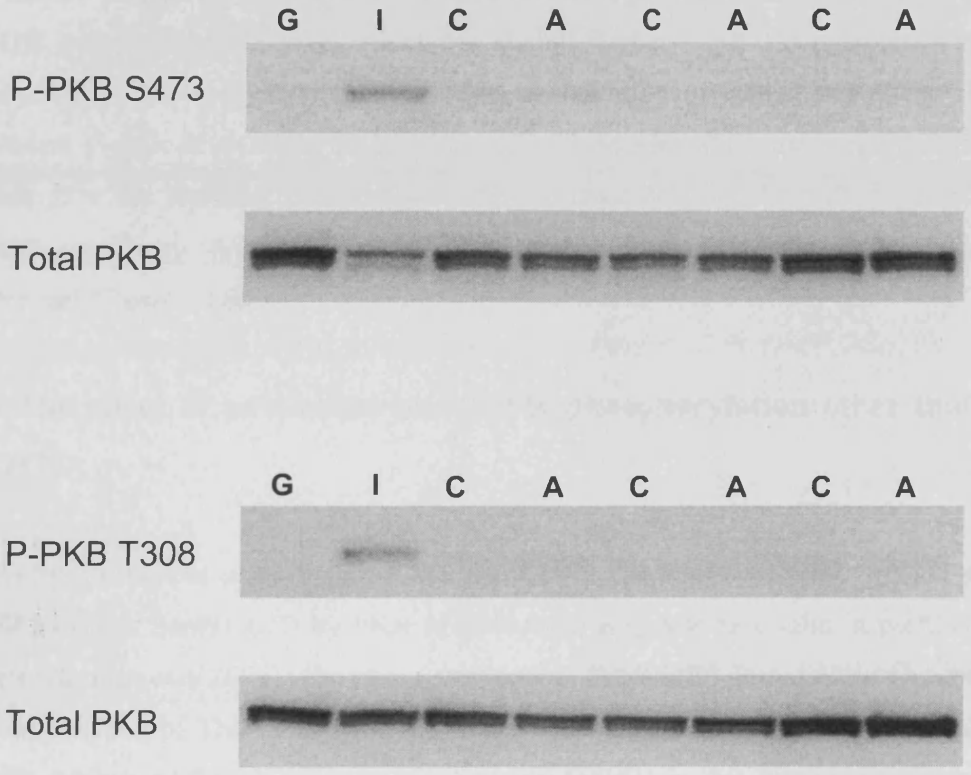


Figure 3.15 The effect of adrenaline on PKB phosphorylation

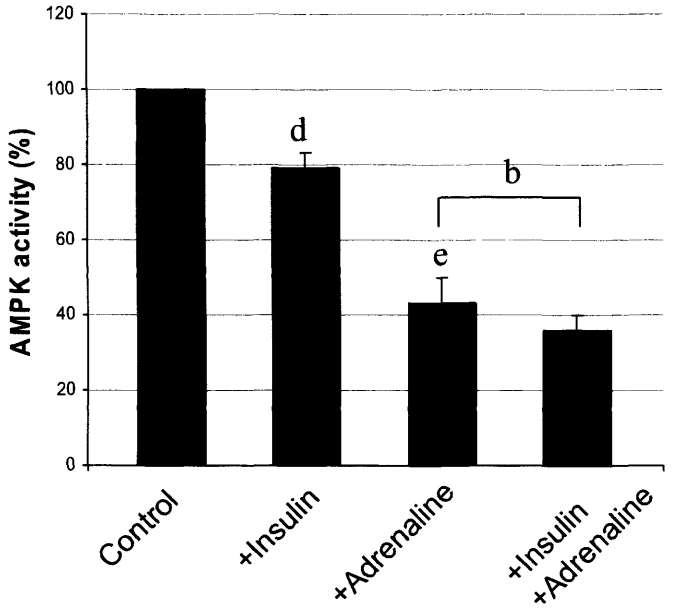
Phosphorylation of PKB Thr308 and Ser473 was examined after incubating cardiac myocytes for 1 hour with 5mM glucose and 0.5mM palmitate in the absence (C) or presence of 2μM adrenaline (A). The figures show the results from three myocyte preparations. As a positive control, PKB phosphorylation was also examined after incubating cells for 1 hour with 5mM glucose in the absence (G) or presence of 10nM insulin (I).

transient activation of PKB by adrenaline through a PI3K-independent pathway. Morisco *et al* reported that isoproterenol caused a PI3K-independent increase in Thr308 phosphorylation which returned to the basal level after 1 hour (Morisco *et al* 2005). However, because the effect of adrenaline on AMPK was sustained for up to 2 hours, a transient activation of PKB is unlikely to account for the effect of adrenaline. Consistent with the idea that adrenaline and insulin inhibit AMPK through distinct pathways, the effect of insulin was found to be additive to that of adrenaline (Figure 3.16). Insulin (10nM) caused an approximately 20% decrease in AMPK activity. The inhibitory effect was slightly higher (~30%) if palmitate was omitted from the incubation, possibly due to inhibition of insulin signalling by palmitate (Soltys *et al* 2002). In the presence of 2μM adrenaline, the concentration which gave the maximal effect of adrenaline (Figure 3.3), insulin still decreased AMPK activity by ~20% (in the presence of palmitate) or ~30% (in the absence of palmitate) (Figure 3.16).

3.6 The effect of adrenaline on AMPK phosphorylation other than Thr172

Recently, Horman *et al* have shown that the AMPK α subunit is phosphorylated at Ser485 (α1) or Ser491 (α2) by PKB *in vitro* or in response to insulin in perfused hearts (Horman *et al* 2006). The phosphorylation of Ser485/491 was shown to inhibit phosphorylation of Thr172 and this was suggested to be the mechanism by which insulin inhibits AMPK (Horman *et al* 2006). Ser485/491 is also phosphorylated by PKA *in vitro* (personal communication from Richard Heath and David Carling). This may explain the inhibitory effect of adrenaline seen in the present study, at least for the effect of the β receptor. Therefore, the effect of adrenaline on Ser485/491 phosphorylation was investigated using a commercially available antibody against this phosphorylation. This antibody was previously used successfully to detect an increase in Ser485/491 phosphorylation by PKB (Soltys *et al* 2006). It was found adrenaline did not significantly increase the phosphorylation of Ser485/491 in cardiac myocytes (Figure 3.17). In addition to Thr172 and Ser485/491, AMPK α and β subunits are known to be phosphorylated at multiple other sites (Section 1.6.4) and the effect of adrenaline may involve phosphorylation of a site other than Ser485/491.

(a)



(b)

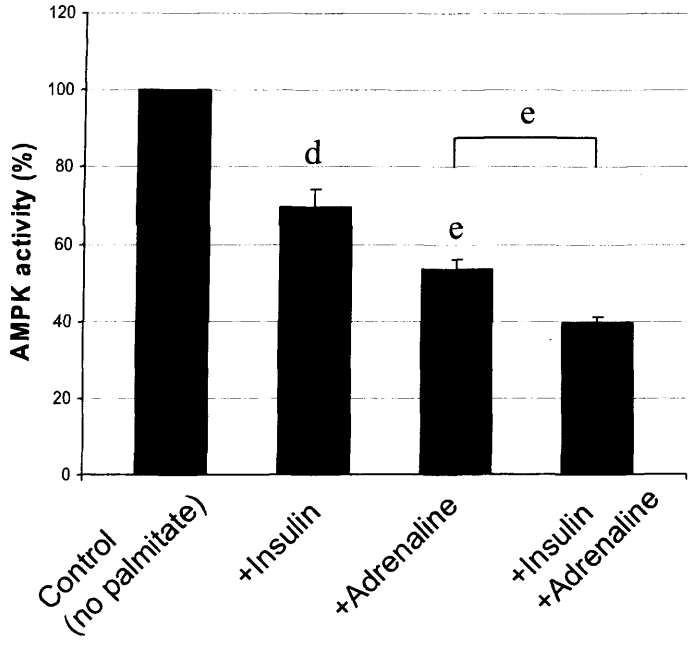
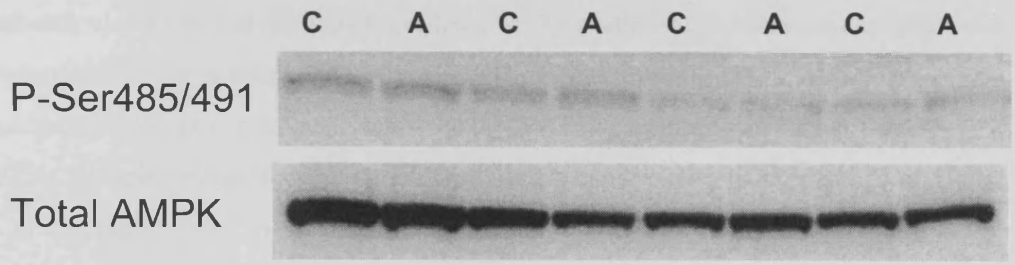


Figure 3.16 The additivity of inhibitory effects of adrenaline and insulin on AMPK

a) Cardiac myocytes were incubated for 1 hour with 5mM glucose and 0.5mM palmitate in the absence or presence of insulin (10nM), adrenaline (2μM), or insulin + adrenaline. α2 AMPK activity was assayed in the presence of 200μM AMP. The results are expressed as percentage of AMPK activity of cells incubated without insulin or adrenaline. n=5 (b) Cardiac myocytes were incubated under similar conditions to (a) except that palmitate was omitted from the incubation medium. n=4

(a)



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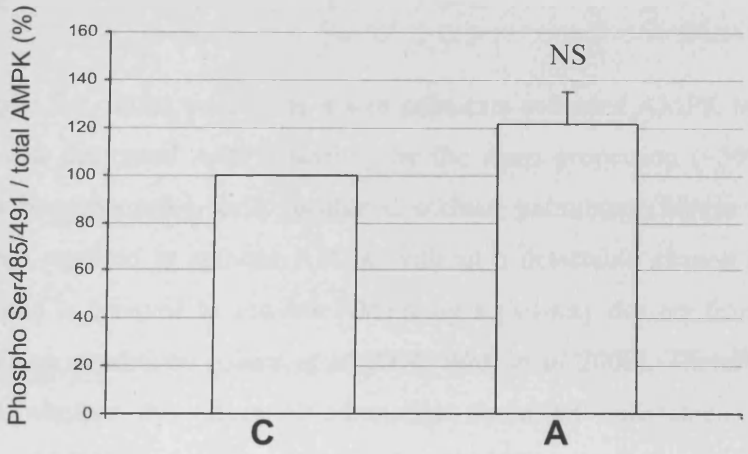


Figure 3.17 The effect of adrenaline on AMPK Ser485/491 phosphorylation

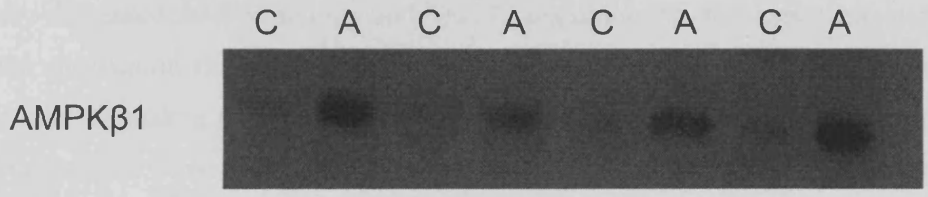
(a) Phosphorylation of AMPK Ser485/491 was examined after incubating cardiac myocytes for 1 hour with 5mM glucose and 0.5mM palmitate in the presence (A) or absence of 2μM adrenaline (C). The figure shows the results from four myocyte preparations. (b) shows band intensity of phospho-Ser485/491 corrected for total AMPKα amount and expressed as percentage of the value of cells incubated without adrenaline. n=8

This possibility was investigated in collaboration with Richard Heath and David Carling from MRC Clinical Sciences Centre, Hammersmith Hospital, Imperial College London. Cell lysates of myocytes treated with or without adrenaline were incubated with recombinant catalytically inactive AMPK ($\alpha1\beta1\gamma1$) in the presence of radioactive ATP. The autoradiograph (Figure 3.18) revealed that the AMPK β1 subunit was more heavily phosphorylated in the lysate of the adrenaline-treated cells compared to the non-treated cells, suggesting that some kinase downstream of the adrenergic receptor phosphorylates the AMPK β1 subunit. Apart from Ser485/491, PKA phosphorylates the β1 subunit at Ser24 *in vitro* (Richard Heath and David Carling, unpublished work). However, phosphorylation of β1 Ser24 was found not to be increased by adrenaline (Figure 3.18).

3.7 Adrenaline selectively attenuates activation of AMPK by sorbitol and palmitate

As shown in Figure 3.3, under conditions where palmitate activated AMPK by 2.5-fold, adrenaline still decreased AMPK activity by the same proportion (~50%) as seen under basal conditions (i.e. cells incubated without palmitate) (Figure 3.16). Palmitate has been reported to activate AMPK without a detectable change in the AMP/ATP ratio and is believed to activate AMPK by a pathway distinct from that used by AMP-raising conditions (Clark *et al* 2004, Watt *et al* 2006). Therefore, it was investigated whether the effect of adrenaline could be maintained under conditions where AMPK was activated by other AMPK-activating stimuli i.e. AICAR (a precursor of the AMP mimetic ZMP), oligomycin (an inhibitor of the F_1F_0-ATPase), phenformin (an inhibitor of complex I of the respiratory chain), and sorbitol (induces hyperosmotic stress). Sorbitol-induced hyperosmolarity has been reported to activate AMPK by an unidentified mechanism that does not involve a change in the AMP/ATP ratio (Fryer *et al* 2002). In this study 100mM sorbitol was used instead of 500mM, which is commonly used to induce hyperosmotic stress (Fryer *et al* 2002), because this concentration was found to be sufficient to cause activation of AMPK and because incubation of the cells with 500mM sorbitol for 1 hour caused a significant loss of myocyte viability. Concentrations of sorbitol similar to 100mM have been previously shown to cause AMPK activation or other

(a)



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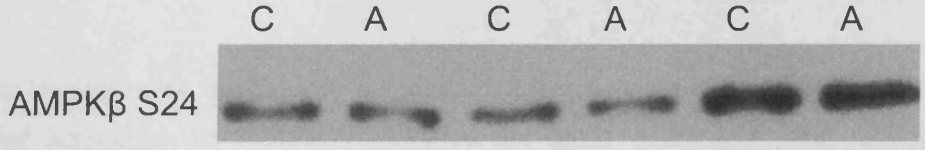


Figure 3.18 The effect of adrenaline on AMPK β-subunit phosphorylation

(a) Cardiac myocytes were incubated for 1 hour with 5mM glucose and 0.5mM palmitate in the presence (A) or absence (C) of 2μM adrenaline. Cell lysates were incubated with recombinant catalytically inactive AMPK α1β1γ1 complex and phosphorylation of AMPK was detected by autoradiography. Only phosphorylation of β1 subunit was increased by incubation with cell lysates. (b) shows western blot analysis of Ser24 phosphorylation of AMPKβ subunit with cell lysates from cells treated with or without adrenaline. The figures show the results from four (a) or three (b) myocyte preparations. The *in vitro* phosphorylation experiment and western blotting were performed by Richard Heath, MRC Clinical Sciences Centre, Hammersmith Hospital, Imperial College London.

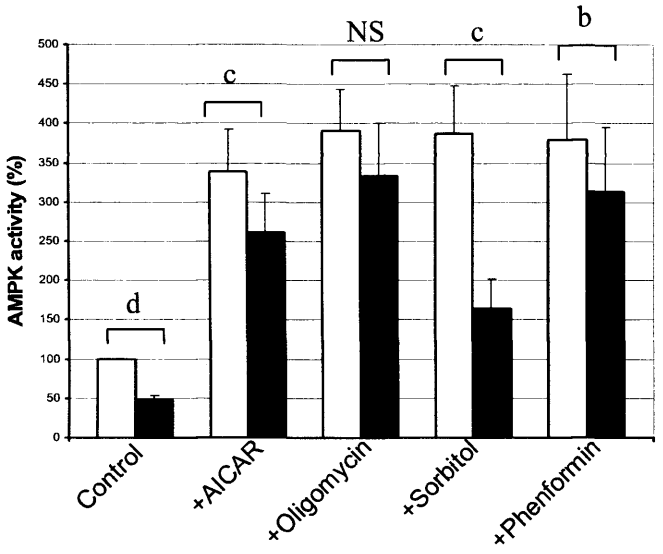
hyperosmotic responses in skeletal muscle and endothelial cells (Fujii *et al* 2005, Ochi *et al* 2002). As shown in Figure 3.19, AICAR, oligomycin, sorbitol and phenformin all activated AMPK to a similar extent (3- to 4-fold). The inhibitory effects of adrenaline on AMPK activity and Thr172 phosphorylation were either greatly diminished or became statistically insignificant in the presence of AICAR, oligomycin or phenformin. In contrast, in the presence of sorbitol, adrenaline still markedly decreased AMPK activity and Thr172 phosphorylation by approximately 50%, the observation similar to that seen in the presence of palmitate. These results suggest that adrenaline may selectively attenuate activation of AMPK by palmitate and hyperosmolar stress. However, as mentioned above, the possibility cannot be ruled out that the effect of adrenaline to attenuate the activation of AMPK by these conditions is mechanistically distinct from the effect of adrenaline seen under basal conditions.

3.8 Adrenergic signalling components mediating the effect of adrenaline

In order to gain more insight into the signalling pathway mediating the inhibitory effect of adrenaline on AMPK activity (under basal conditions), the possible involvement of several well-recognised adrenergic signalling components was examined. Incubation of the cells with forskolin or the cell permeable cAMP analogue dibutyryl cAMP did not significantly affect AMPK activity (Figures 3.20 and 3.21). Forskolin and dibutyryl cAMP activated PKA as evidenced by the increased PKA-driven phosphorylation of PLB Ser16. AMPK activity was also unaffected by 8-CPT-2'-O-Me-cAMP, a selective activator of Epac (Enserink *et al* 2002). These results exclude the involvement of cAMP signalling or PKA in the effect of adrenaline.

CAMKII has been shown to be activated by the β receptor in quiescent cardiac myoctes through a PKA-independent activation of the L-type Ca^{2+} channel, possibly by direct activation of the Ca^{2+} channel by G_s (Zhu *et al* 2003). Because CAMKII is also known to be activated by the α_1 receptor (Wang *et al* 2001), the involvement of

(a)



(b)

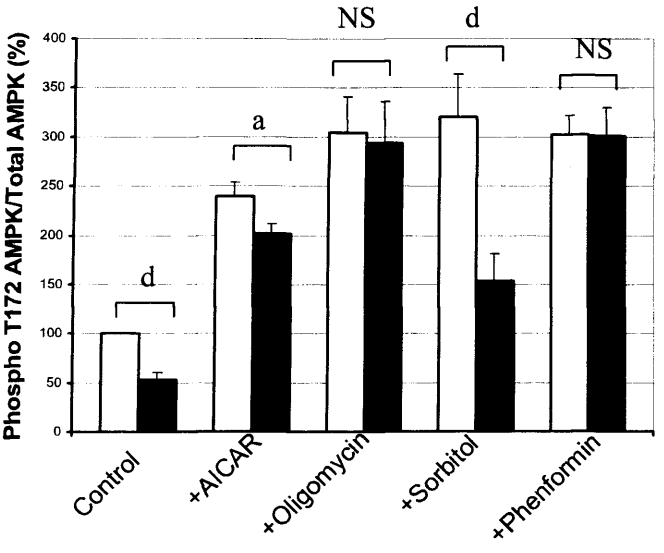
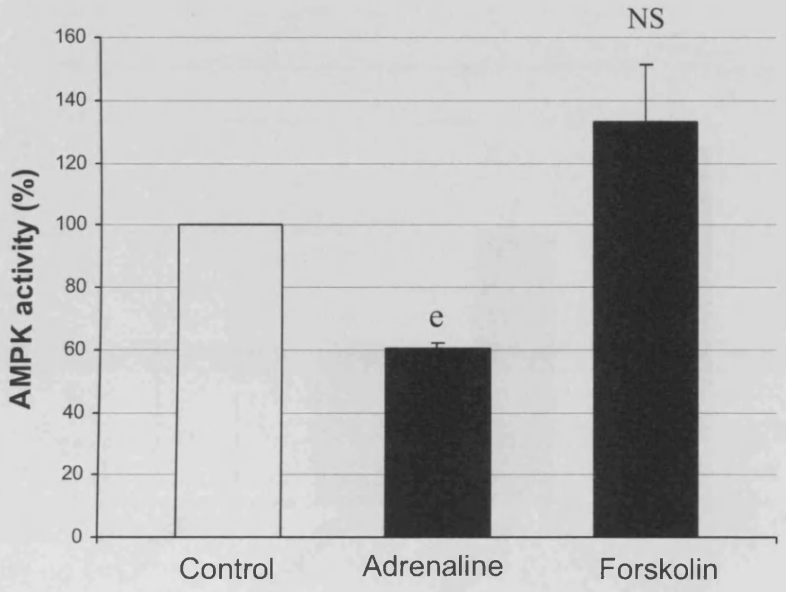


Figure 3.19 The effect of adrenaline on activation of AMPK by different conditions

α2 AMPK activity (a) and Thr172 phosphorylation (b) were measured after incubating cardiac myocytes for 1 hour with 5mM glucose and 0.5mM palmitate alone (control), or with 1mM AICAR, 5μM oligomycin, 100mM sorbitol, or 10mM phenformin, in the presence (filled bar) or absence (open bar) of 2μM adrenaline. AMPK activity was assayed in the presence of 200μM AMP. The band intensity of Thr172 phosphorylation was quantified and corrected for total AMPKα. AMPK activity and Thr172 phosphorylation are expressed as percentage of the values of cells incubated without adrenaline and AMPK-activating agents. AMPK activity of control cells was 29.8±12.3pmol/min/mg protein. n=4

(a)



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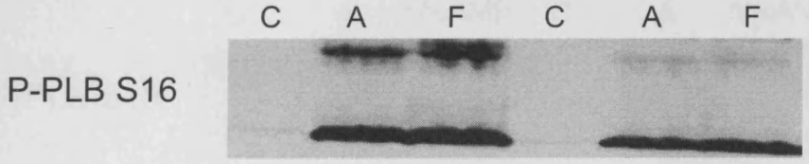
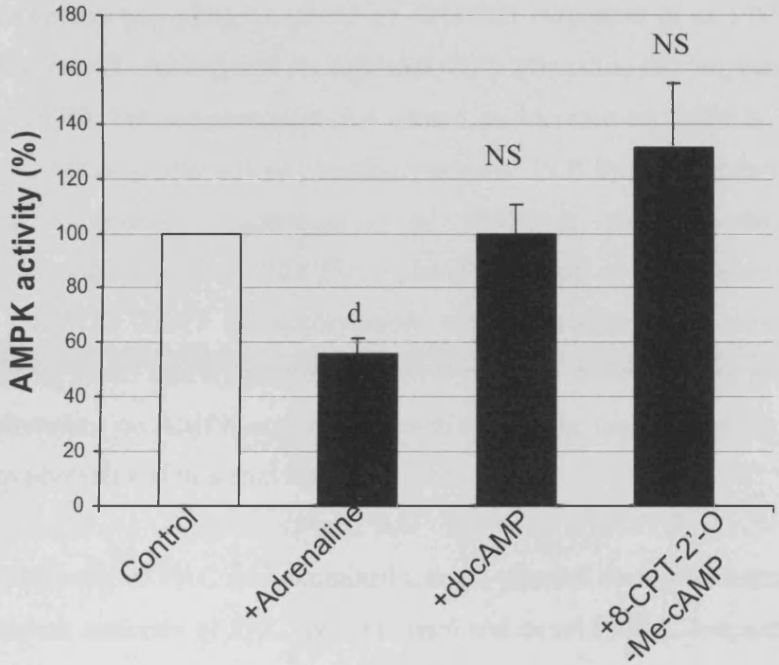


Figure 3.20 The effect of forskolin on AMPK activity

(a) α2 AMPK activity was assayed in the presence of 200μM AMP after incubating cardiac myocytes for 1 hour with 5mM glucose and 0.5mM palmitate alone (control) or with 2μM adrenaline or 10μM forskolin. The results are expressed as percentage of AMPK activity of cells incubated without adrenaline or forskolin. n=4 (b) Activity of PKA in control cells (C), adrenaline-treated cells (A), and forskolin-treated cells (F) were checked with phosphorylation of PLB Ser16. The figure shows the results from two myocyte preparations.

(a)



(b)

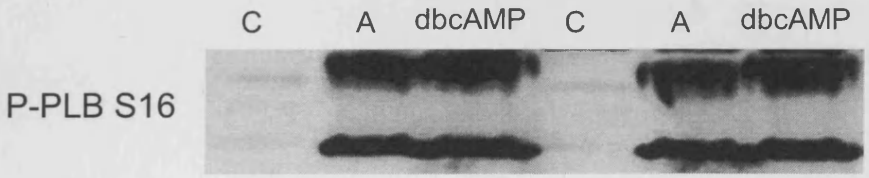


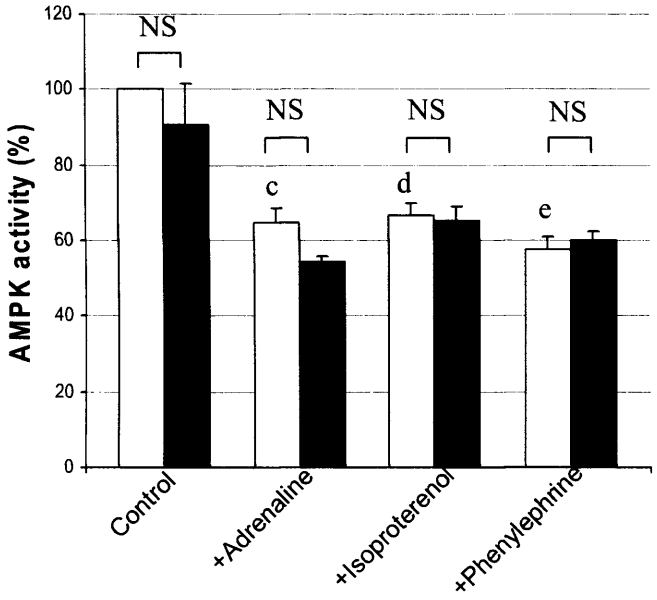
Figure 3.21 The effect of cAMP analogues on AMPK activity

(a) α2 AMPK activity was assayed in the presence of 200μM AMP after incubating cardiac myocytes for 1 hour with 5mM glucose and 0.5mM palmitate alone (control) or with 2μM adrenaline, 1mM dybutyryl cAMP, or 50μM 8-CPT-2'-O-Me-cAMP. The results are expressed as percentage of AMPK activity of cells incubated without adrenaline or cAMP analogues. n=4 (b) Activity of PKA in control cells (C), adrenaline-treated cells (A), and cells treated with dybutyryl cAMP (dbcAMP) were checked with phosphorylation of PLB Ser16. The figure shows the results from two myocyte preparations.

this enzyme in the effect of adrenaline was investigated. The effect of adrenaline, isoproterenol, or phenylephrine on AMPK activity was unaffected by 10μM KN93 (Figure 3.22). In order to check that this concentration of the inhibitor was sufficient to block CAMKII activation, the effect of the inhibitor on posphorylation of PLB Thr17 (the site exclusively phosphorylated by CAMKII (Wegener *et al* 1989)) by adrenergic agonists was investigated. As expected, both adrenaline and isoproterenol (Kuschel *et al* 1999), but not phenylephrine caused an increase in the PLB Thr17 phosphorylation. Although the lack of phosphorylation of PLB by phenylephrine has been observed previously (Lindemann *et al* 1986), it was observed that phenylephrine actually decreased PLB Thr17 phosphorylation in the present study. The increase in PLB Thr17 phosphorylation with adrenaline was completely inhibited by KN93 while that by isoproterenol was partially inhibited. The fact that the effect of adrenaline on AMPK activity was seen despite the blockade of CAMKII rules out the involvement of this enzyme.

A possible involvement of PKC was examined using a phorbol ester which activates the DAG-dependent isoforms of PKC (conventional and novel PKCs). Incubation of myocytes with 1μM PMA completely mimicked the effect of adrenaline (Figure 3.23), suggesting the possibility that PKC mediates the effect of adrenaline.

(a)



(b)

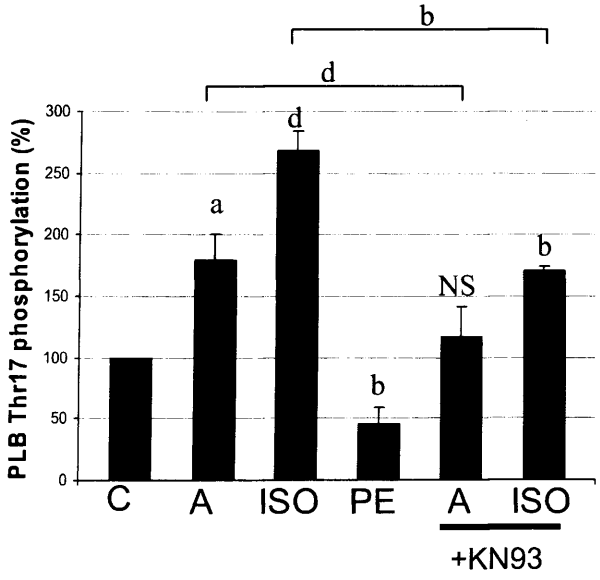


Figure 3.22 The effect of KN93 on the effect of adrenaline on AMPK

(a) Cardiac myocytes were pre-incubated with 5mM glucose and 0.5mM palmitate in the presence (filled bar) or absence (open bar) of 10μM KN93 for 30 minutes followed by further 30 minutes incubation under control condition (no addition), or with 2μM adrenaline, 2μM isoproterenol + 2μM phentolamine, or 100μM phenylephrine + 2μM propranolol. α2 AMPK activity was assayed in the presence of 200μM AMP and results were expressed as percentage of AMPK activity of cells incubated without KN93 or adrenergic agonists. n=3-4 (b) The effects of adrenaline (A), isoproterenol (ISO), phenylephrine (PE), and KN93 on phosphorylation of PLB Thr17 are shown. n=3

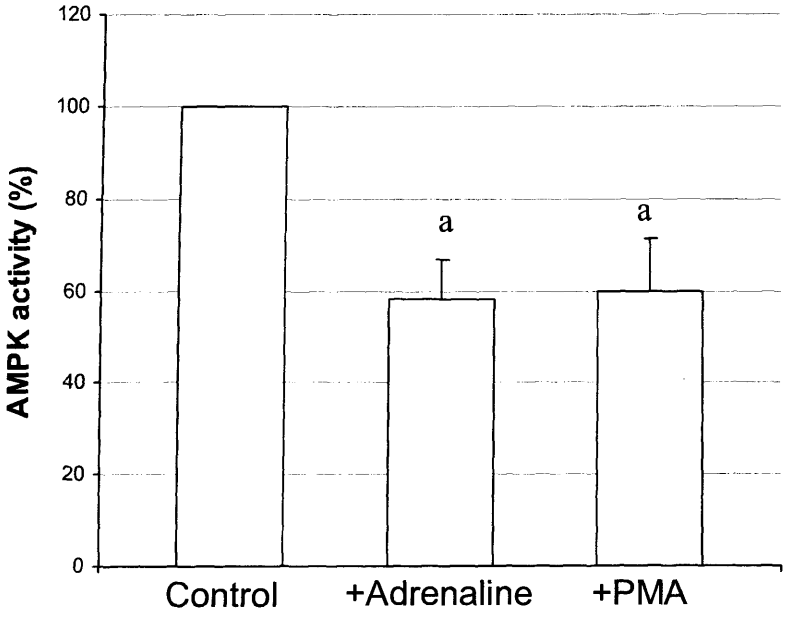


Figure 3.23 The effect of PMA on AMPK activity

Cardiac myocytes were incubated for 1 hour with 5mM glucose and 0.5mM palmitate alone (control) or with 2μM adrenaline or 1μM PMA. α2 AMPK activity was assayed in the presence of 200μM AMP and the results were expressed as percentage of AMPK activity of cells incubated without adrenaline or PMA. n=3

3.9 General Discussion

A previous study with perfused hearts found that adrenaline could decrease AMPK activity only when AMPK was activated by palmitate, suggesting an indirect effect of adrenaline on cardiac AMPK (Clark *et al* 2004). The lack of a direct effect of adrenaline on AMPK activity in perfused hearts was confirmed in the present study. However, in isolated cardiac myocytes, adrenaline unexpectedly decreased AMPK activity and Thr172 phosphorylation even in the absence of palmitate. This effect is unlikely to be an experimental artefact caused by toxic effects of adrenaline, since the decrease in AMPK activity was not associated with a decrease in viability, total AMPK and ACC protein amount, or the total amount of adenine nucleotides and creatine compounds. As discussed below, there are several potential explanations for this direct inhibitory effect of adrenaline on AMPK in myocytes and the lack of this effect in perfused hearts.

1. While adrenaline stimulates both energy production and contractile activity in perfused hearts, in quiescent myocytes, adrenaline is likely to increase energy production without significantly increasing energy expenditure. Thus, it is possible that adrenaline inactivates AMPK in quiescent myocytes by decreasing the AMP/ATP ratio. This effect may be more pronounced if myocytes are suffering from low cellular levels of ATP and CrP, possibly due to cell damage sustained during the isolation procedure or due to other conditions of the incubation (e.g. hypoxia), and if AMPK activity is already significantly activated under basal conditions. The values of ATP and CrP in myocytes obtained in this study (~10nmol/mg proteins) are rather low compared to ~20nmol/mg protein reported by other investigators (Geisbuhler *et al* 1984; Ladilov *et al* 2003). However, the AMP/ATP ratio obtained in myocytes in this study is comparable to values obtained by other investigators (Luiken *et al* 2003). In the present study, in order to obtain clearly visible peaks (especially for AMP) by HPLC, concentrated samples were prepared by extracting the cells with a minimal volume of 0.21M PCA. It was found difficult to obtain concentrated neutralised extracts in sufficient amount with higher concentrations of PCA because of increased precipitation. However, this concentration of PCA is lower than the concentrations commonly used to extract cells, and a previous study measuring the

high energy phosphates using the same method as used in this study reported incomplete extraction of ATP by 0.21M PCA (Sellevold *et al* 1986). It was also found in the present study that values of adenine nucleotides in the perfused hearts measured in the extracts prepared by 0.21M PCA were also approximately half of the values obtained previously using a different method, despite the comparable AMP/ATP and ATP/ADP ratios. Thus, the low amount of high energy phosphates seen in the myocytes in this study probably reflects an incomplete extraction of adenine nucleotides and creatine compounds rather than energy-deprivation of the myocytes. Whether the myocytes were energy-depleted or not, the observations that both AMP/ATP and Cr/CrP ratios were unaltered by adrenaline seems to exclude the possibility that adrenaline decreases AMPK activity by increasing ATP production. The incomplete extraction of the adenine nucleotides and creatine compounds is a systematic error and would be unlikely to affect this conclusion. However, the possibility that adrenaline caused a small or localised change in free AMP and/or ATP which could not be detected with the method used in the present study cannot be ruled out. *In vitro*, AMPK has been reported to be allosterically activated or inhibited by NAD^+ or NADH, respectively (Rafaeloff-Phail *et al* 2004). Thus, the effect of adrenaline might also involve a change in NAD^+/NADH, although the effect of NAD^+/NADH on AMPK Thr172 phosphorylation is unknown.

2. Adrenaline increases contractile activity in perfused heart. It is possible that there is a direct inhibitory effect of adrenaline on AMPK in cardiac myocytes (isolated or in the whole heart), but in perfused hearts, this effect may be suppressed by some signalling process which is induced by contraction. For example, one possible signalling process which might be antagonistic to the inhibitory effect of adrenaline is PI3K, which has been reported to be activated by electrically-induced contraction in cardiac myocytes (Till *et al* 2000). This contraction-activated PI3K is likely to be different from the PI3K isoform or the pool of PI3K which is involved in the inactivation of AMPK by insulin because its activation is not associated with activation of PKB (Till *et al* 2000). In endothelial cells, activation of AMPK by metformin appears to be mediated by peroxynitrite which activates the cSrc-PI3K-PDK1-PKCζ pathway and increases association of LKB1 with AMPK, possibly by PKCζ phosphorylation of LKB1 (Section 1.6.5.g). In the heart, metformin has also been reported to increase the association of AMPK with LKB1 (Zou *et al* 2004).

Moreover, it has been shown peroxynitrite activates AMPK in cardiac myocytes and the effect of metformin on AMPK in cardiac myocytes is abolished by inhibiting peroxynitrite (Zou *et al* 2004). Taken together, these results suggest the possibility that in cardiac myocytes, there are two pools/isoforms of PI3K which have opposite effects on AMPK. One of these is inhibitory, involves PKB, and is activated by insulin. The other has an activating effect, does not involve PKB, and is activated by peroxynitrite. It is possible that the isoform/pool of PI3K which activates AMPK is activated by contraction and this may counter the inhibitory effect of adrenaline on AMPK.

3. It was observed that adrenaline could decrease AMPK activity to the same extent as seen under basal conditions when AMPK was activated by palmitate or sorbitol. In contrast, the inhibitory effect of adrenaline was either markedly diminished or became statistically insignificant under conditions where AMPK was activated by AICAR, oligomycin, or phenformin, the conditions which activate AMPK by the AMP-pathway. These results raise the possibility that the inhibitory effect of adrenaline seen under basal conditions in myocytes is due to blockade of tonic activation of AMPK through the pathway which is activated by palmitate or hyperosmotic stress. Both palmitate and sorbitol-induced hyperosmolarity have been reported to activate AMPK by a pathway distinct from the AMP-raising conditions (Fryer *et al* 2002; Daniel and Carling 2002b; Clark *et al* 2004; Watt *et al* 2006). It is not known whether these two conditions activate AMPK by the same mechanism, although the present observation that adrenaline selectively attenuated activation of AMPK by these conditions support this possibility. The tonic activation of AMPK through the pathway which is activated by palmitate/hyperosmolarity would be expected to be dwarfed under conditions where AMPK is activated through other pathways (e.g. the AMP-pathway), explaining the observation that the inhibitory effect of adrenaline was blunted in the presence of AICAR, oligomycin, or phenformin. Although oligomycin and phenformin have been shown to increase cellular AMP (Luiken *et al* 2003; Hawley *et al* 2005), the possibility that these agents activated AMPK without increasing AMP cannot be ruled out, given the reports that hypoxia in perfused hearts and metformin (a compound related to phenformin) activate AMPK by AMP-independent mechanisms (Altarejos *et al* 2005; Frederich *et al* 2005; Hawley *et al* 2002; Fryer *et al* 2002; Zou *et al* 2003 and

2004). However, it is still possible that the AMP-independent activation of AMPK by oligomycin and phenformin is mechanistically distinct from activation of AMPK by palmitate or sorbitol and is not blocked by adrenaline. It was observed that the AMP/ATP ratio in perfused hearts was significantly higher ($P<0.05$, unpaired test) than that in myocytes (the AMP/ATP ratios of perfused hearts and myocytes fuelled with 5mM glucose and 0.5mM palmitate for 1 hour were 0.087±0.002 (n=4) and 0.045±0.010 (n=8), respectively). This observation has been made by other investigators (Geisbuhler *et al* 1984) and it is probably due to the lack of contractile activity in myocytes. Supporting this, Luiken *et al* have shown that electrically-induced contraction in quiescent cardiac myocytes caused an approximately 60% increase in AMP (Luiken *et al* 2003). The lack of the significant inhibitory effect of adrenaline in perfused heart under basal conditions may be explained by the higher AMP/ATP ratio and the basal activation of AMPK through the AMP pathway which masks the effect of adrenaline on the tonic activation of AMPK though the palmitate/sorbitol pathway.

4. Lastly, the inhibitory effect of adrenaline on basal AMPK activity in myocytes may be due to blockade of activation of AMPK by some unknown condition during the incubation that activated AMPK through the same pathway as sorbitol/palmitate or through other pathways which are also inhibited by adrenaline.

Whatever the actual reason for the lack of a direct inhibitory effect of adrenaline in perfused hearts, the present data at least suggest the possibility of an adrenergic signalling pathway that directly regulates AMPK in cardiac myocytes.

The effect of adrenaline was found to be mediated by both α_1 and β adrenoreceptors. In contrast to this finding, An *et al* have reported no significant effect of 10μM isoproterenol on AMPK Thr172 phosphorylation in cardiac myocytes (An *et al* 2005). The reason for this discrepancy is not clear. The involvement of both receptor types in an effect of adrenaline is not unprecedented. Several signalling or downstream effects of adrenaline, such as Ca^{2+} signalling, MAPK activation, and glucose transport, have been reported to be stimulated by both receptors (Sections 1.5.3, 1.5.9, and 1.4.2). Although glucose has been reported to inactivate AMPK by an unknown mechanism that does not involve a change in the AMP/ATP ratio in

skeletal muscle (Itani *et al* 2003; Halse *et al* 2003), the inhibitory effect of adrenaline observed in the present study is unlikely to be secondary to adrenergic stimulation of glucose uptake/metabolism because the effect of adrenaline on AMPK was seen in the absence of glucose. Cardiac myocytes express several subtypes of α_1 and β receptors including α_{1A}, α_{1B}, α_{1D}, β_1, β_2 and β_3 receptors (Section 1.2). While the β_1 receptor is exclusively coupled to the G_s-cAMP pathway, the β_2 and possibly β_3 receptors are additionally coupled to G_i signalling (Section 1.3). Although the three α_1 receptor subtypes are all coupled to G_q, stimulation of different α_1 receptor subtypes has been reported to cause distinct downstream effects, possibly due to coupling to additional G proteins and compartmentalisation of the receptors (Section1.4.3). Further study is required to determine the adrenergic receptor subtypes and G proteins involved in the effect of adrenaline.

Insulin has been reported to inhibit cardiac AMPK *via* the PI3K-PKB pathway (Beauloye *et al* 2001; Kovacic *et al* 2003). Because PI3K has also been reported to be activated by cardiac adrenergic receptors, PI3K-PKB became a likely candidate for the pathway responsible for the inhibitory effect of adrenaline. However, the observations that PKB was not activated by adrenaline and the effect of adrenaline was unaffected by PI3K inhibition excluded this possibility. The inhibitory effect of insulin has recently been reported to be due to PKB phosphorylation of Ser485/491 of AMPK α subunit, which prevents phosphorylation of Thr172 by LKB1 and possibly by other AMPKKs (Horman *et al* 2006; Soltys *et al* 2006). Recently, this site has been reported to be also phosphorylated by PKA *in vitro* (Hurley *et al* 2006). However, Ser485/491 phosphorylation was not increased by adrenaline in cardiac myocytes, indicating this phosphorylation is not responsible for the effect of adrenaline. The effect of adrenaline also does not involve cAMP signalling, PKA, or CAMKII. The observation that the effect of adrenaline was mimicked by PMA may suggest the involvement of PKC. PKC is a well-established component of α_1 adrenergic signalling. Activation of PKC in response to stimulation of the β receptor has also been reported in adult rat cardiac myocytes (Shizukuda and Buttrick 2001), although the mechanism by which the β receptor activates PKC is unknown. Thus, PKC may be involved in both α_1- and β-mediated effects of adrenaline on AMPK. Adult rat cardiac myocytes appear to express at least PKCε and PKCζ, and possibly PKCα, PKCβ, and PKCδ (Malhotra *et al* 2001b; Bogoyevitch *et al* 1993; Puceat *et al*

1994). However, stimulation of cardiac α_1 or β receptors has been reported to selectively activate PKCε and possibly PKCδ (novel PKCs) without affecting conventional and atypical PKCs (Puceat *et al* 1999; Clerk *et al* 1994; Wang *et al* 2003; Deng *et al* 1998; Shizukuda and Buttrick *et al* 2001). In contrast, in cardiac myocytes, PMA (a DAG analogue) has been shown to activate both conventional (PKCα) and novel PKCs (PKCε and PKCδ) (Puceat *et al* 1994). Phorbol esters are also known to activate other enzymes such as PKDs, which are also DAG dependent, PI3K and PLD (van Lint 1995; Farese 2002). Therefore, although the finding that PMA decreased AMPK activity in cardiac myocytes is an interesting observation and warrants further research, involvement of PKC in α_1 and β mediated effect of adrenaline needs to be examined using selective PKC inhibitors.

At present, how adrenaline signalling brings about a decrease in Thr172 phosphorylation is unknown. In principle, the effect of adrenaline could involve inhibition of AMPKKs or stimulation of dephosphorylation by protein phosphatases. The heart expresses LKB1, CAMKKβ (but not α) and possibly other, as yet unidentified AMPKKs (Sakamoto *et al* 2006; Allard *et al* 2006; Baron *et al* 2005; Altarejos *et al* 2005). Further study is necessary to determine if adrenaline affects the activities of any of these AMPKKs. Alternatively, adrenaline could affect Thr172 phosphorylation by a direct covalent modification of AMPKKs or AMPK, which prevents Thr172 phosphorylation by a mechanism other than reduction in AMPKK catalytic activity. For example, adrenaline could decrease Thr172 phosphorylation by preventing the association of AMPK with AMPKKs, by making AMPK a better substrate for a protein phosphatase, or by increasing affinity of AMPK for ATP. In this regard, it is of note that adrenaline increased phosphorylation of the AMPK β1 subunit. Further study is required to identify the residue(s) phosphorylated and the role of this phosphorylation in the inactivation of AMPK by adrenaline. A number of phosphorylation sites have been identified on the β1 subunit including Ser24, Ser25, Ser96, Ser101, Ser108, and Ser182 (Mitchelhill *et al* 1997; Woods *et al* 2003; Chen *et al* 1999). It was found adrenaline did not increase phosphorylation of Ser24. Preliminary experiments by Richard Heath also suggested that phosphorylation of Ser108 and Ser182 were not increased by adrenaline. Thus, adrenaline might increase phosphorylation at Ser25, Ser96, Ser101, or an other yet unidentified site.

The β1 subunit contains 21 serine residues and 15 threonine residues (Chen *et al* 1999) which could be potentially phosphorylated.

Although one potential clue to understanding the mechanism of the effect of adrenaline is the observation that the increase in AMPK activity and Thr172 phosphorylation induced by sorbitol or palmitate was markedly inhibited by adrenaline, at present, very little is known about how these conditions increase Thr172 phosphorylation. It has been reported in cultured cells that although LKB1 is required for phosphorylation of Thr172 by hyperosmolarity, catalytic activity of LKB1 is unaffected (Woods *et al* 2003, Shaw *et al* 2003). Thus, like AMP, hyperosmolarity also appears to increase Thr172 phosphorylation by making AMPK a better substrate for LKB1. The facilitation of Thr172 phosphorylation by AMP is thought to be due to a conformational change of the AMPK complex which exposes Thr172 (Cheung *et al* 2000). It is possible that activation of AMPK by hyperosmolarity involves a similar conformational change of AMPK or, as suggested for activation of AMPK by metformin (Zou *et al* 2004), it involves increased association of LKB1 with AMPK. The mechanism by which fatty acids activate AMPK also does not appear to involve stimulation of LKB1 catalytic activity (Watt *et al* 2006), and might also involve making Thr172 a better substrate and/or activation of other AMPKKs.

It appears there is considerable tissue-specific difference in adrenergic signalling and AMPK regulation. In contrast to the present finding that adrenaline inhibits AMPK in cardiac myocytes, adrenergic agents have been reported to increase AMPK activity and/or Thr172 phosphorylation in skeletal muscle cells and adipocytes (Section 1.6.5.p). It has been shown that the activation of AMPK in adipocytes is mediated by the β receptor and is mimicked by cAMP-raising treatments (Yin *et al* 2003; Daval *et al* 2005; Hutchinson *et al* 2005). The mechanism by which cAMP signalling activates AMPK in adipocytes is unknown. However, in liver, glucagon has been shown to increase AMPK Thr172 phosphorylation and this has been suggested to be due to PKA phosphorylation of LKB1 at Ser428 (Kimball *et al* 2004). Phosphorylation of this site has been implicated in the increased association of LKB1 with AMPK (Xie *et al* 2006). Thus, β receptor stimulation in adipocytes may also involve PKA phosphorylation of LKB1. However, in contrast to

adipocytes, stimulation of the β receptor does not activate AMPK in skeletal muscle, indicating cAMP/PKA does not regulate AMPK in skeletal muscle (Minokoshi *et al* 2002). Moreover, a very recent study has reported that cAMP-raising conditions decrease AMPK activity and Thr172 phosphorylation in INS-1, MEF, and COS cells (Hurley *et al* 2006). This effect has been reported to be due to inhibition of the catalytic activity of CAMKKα and β and to PKA phosphorylation of AMPK Ser485/491 (Hurley *et al* 2006). CAMKKβ is also expressed in the heart (Allard *et al* 2006). However, cAMP-raising agents did not affect AMPK activity in cardiac myocytes. Ser485/491 phosphorylation was also found not to be increased by adrenaline despite activation of PKA as evidenced by increased phosphorylation of PLB Ser16. The activation of AMPK in skeletal muscle by noradrenaline has been reported to be mediated by the α_1 receptor (Hutchinson and Bengtsson 2006). α_1 receptor-mediated Ca^{2+} signalling has been implicated in the activation of AMPK in this tissue because the effect was mimicked by a Ca^{2+} ionophore (Hutchinson and Bengtsson 2006). Activation of AMPK by Ca^{2+}-raising treatments, including stimulation of G_q coupled receptors has been observed in several other cell types and it is likely to be due to activation of CAMKKs (Section 1.6.5.h). However, it appears that raising Ca^{2+} does not activate AMPK in cardiac myocytes. Although Ca^{2+} was not measured in the present study, adrenaline increased activity of CAMKII, indicating activation of Ca^{2+} signalling. In the present study, phorbol ester was found to inhibit AMPK activity. However, phorbol ester has been reported to have no effect on AMPK in skeletal muscle (Hutchinson and Bengtsson 2006), while it has been shown to activate AMPK in endothelial cells (Xie *et al* 2006). The effect of phorbol ester in endothelial cells was probably due to activation of PKCζ, possibly *via* activation of PI3K and PDK1 (Farese 2002), as it was abolished by an inhibitor for PKCζ. As mentioned above, there is the possibility that PKCζ also activates AMPK in cardiac myocytes. Although the present observation that PMA inhibited AMPK in cardiac myocytes apparently contradicts this possibility, phorbol ester has been reported to have no effect on the activation of PKCζ in cardiac myocytes (Puceat *et al* 1994). The reason for these tissue-specific effects of adrenergic signalling on AMPK is unknown, but it may be partly due to difference in expression pattern of AKAPs, PKC anchoring proteins, or different compartmentalisation of Ca^{2+} signalling. It could also be due to differences in expression of AMPKKs and other AMPK-associated proteins. The tissue specificity of the hormonal regulation of

AMPK is not unique to adrenergic agents. Both leptin and ghrelin have been shown to have different effects on AMPK in different tissues (Sections 1.6.5.i and j).

It is not clear whether adrenaline decreases cardiac AMPK activity *in vivo*. Although adrenaline appears to have a direct inhibitory effect on AMPK in cardiac myocytes, the observations in perfused hearts made in the present study and in the previous study by Clark *et al* suggest that the activation of AMPK by palmitate is essential for the effect of adrenaline to inactivate AMPK in perfused hearts. However, because the heart is normally exposed to long chain fatty acids *in vivo*, it is possible that adrenaline decreases cardiac AMPK activity *in vivo*. However, at present, there is a considerable controversy in the literature over the effect of long chain fatty acids to activate AMPK. Apart from the previous study by Clark *et al*, the activation of AMPK by long chain fatty acids has been reported in cultured neonatal cardiac myocytes and in skeletal muscle cells (Hickson-Bick *et al* 2000; Fediuc *et al* 2006; Watt *et al* 2006). However, other investigators have failed to see an activation of AMPK by long chain fatty acids in perfused hearts or in rat skeletal muscle (Crozier *et al* 2005; Folmes *et al* 2006; Olsen and Hansen 2002). Crozier *et al* have even reported a decrease in AMPK Thr172 phosphorylation in hearts perfused with palmitate compared to hearts perfused with glucose alone (Crozier *et al* 2005). An *in vivo* study showed that dietary polyunsaturated fatty acids decreased the extent of a decrease in rat hepatic AMPK Thr172 phosphorylation and activity seen during fasting/refeeding (Suchankova *et al* 2005), implying activating effect of fatty acids. In contrast, a study by Dobrzyn *et al* in mice showed that dietary polyunsaturated fatty acids did not cause any change in AMPK Thr172 phosphorylation in liver, skeletal muscle, and heart (Dobrzyn *et al* 2005). The lack of the effect of fatty acids to activate AMPK may explain the observation by King *et al* that infusion of dobutamine did not decrease cardiac AMPK activity *in vivo* (King *et al* 2005). In the present study, although a significant 2- to 3- fold activation of AMPK by palmitate was observed in cardiac myocytes in the early stage of the study, this marked effect was subsequently lost for unknown reasons. Palmitate also did not activate AMPK in perfused hearts indicating the loss of the effect is not related to myocyte isolation. It is still possible that hearts perfused with glucose alone were energetically compromised, and the AMPK-activating effect of palmitate was blunted by a simultaneous effect of palmitate to increase ATP production and so inhibit AMPK.

However, this is unlikely because HPLC analysis of the total adenine nucleotides showed that the AMP/ATP ratio in the hearts perfused with glucose alone (0.067±0.02, n=4) was not significantly different from that in the hearts perfused with palmitate (0.087±0.02, n=4).

The observation that adrenaline inhibited activation of AMPK by sorbitol in myocytes suggests that adrenaline may inhibit cardiac AMPK *in vivo* during hyperosmotic situations. However, it is not known whether this inhibitory effect of adrenaline on hyperosmolarity-induced activation of AMPK holds in the heart, and whether relatively small physiological fluctuations in blood osmolarity affect cardiac AMPK activity significantly.

Studies in cardiac myocytes and skeletal muscle have suggested that activation of AMPK attenuates protein translation through activation of eEF2 kinase and through inhibition of the mTOR-p70 S6 kinase pathway (Chan and Dyck 2005). Moreover, activation of AMPK by AICAR or adiponectin has been shown to inhibit α_1 adrenergic receptor-induced hypertrophic response in cardiac myocytes (Chan *et al* 2004; Shibata *et al* 2004). Therefore, the inhibition of AMPK by adrenaline could potentially play a role in the development of hypertrophy by prolonged adrenergic stimulation. It appears that AMPK has an anti-apoptotic role in the heart (Terai *et al* 2005; An *et al* 2006). Thus, the effect of adrenaline to inhibit AMPK might also be involved in adrenergic stimulation of apoptosis. Both AMPK and adrenergic stimulation regulate some of the key enzymes/processes involved in glucose and fatty acid catabolism, namely glucose uptake, PFK2, and ACC2. Cardiac ACC2 has been shown to be phosphorylated and inactivated by PKA and AMPK *in vitro* (Boone *et al* 1999). Although phosphorylation of ACC has been shown to occur in cardiac myocytes in response to isoproterenol (Boone *et al* 1999), it has been reported that adrenaline does not change ACC activity in perfused hearts (Goodwin and Taegtmeyer 1999). This observation may be explained by the inhibition of AMPK activity by adrenaline. In hearts exposed to fatty acids (e.g. in the above study by Goodwin and Taegtmeyer and *in vivo* hearts), it is possible that there is high basal phosphorylation of ACC2 by AMPK due to activation of AMPK by fatty acids. Adrenergic stimulation increases PKA-driven phosphorylation of ACC2, but at the same time removes the inhibitory effect of ACC2 by AMPK by inactivating AMPK.

This might result in no net change in ACC2 activity. The inhibition of AMPK by adrenaline is contradictory to the effect of the hormone to stimulate glucose catabolism in the heart. Similar paradoxical effects are also seen for insulin, which has been reported to inactivate cardiac AMPK but is also known to stimulate glucose uptake and glycolysis (Folmes *et al* 2006). It may be possible that the activating effect of adrenaline on glucose catabolism (e.g. through PKA and Ca^{2+}-dependent mechanisms) dominates over the inhibitory effect due to removal of the activating effect of AMPK. Alternatively, there may be mechanisms to ensure that glucose catabolism is increased by adrenaline despite inhibition of AMPK. For example, the pool of AMPK that regulates glucose uptake and glycolysis may not be inhibited by adrenaline due to compartmentalisation of adrenergic signalling. The effect of adrenaline to inhibit AMPK may actually be a mechanism to fine-tune fuel catabolism according to the energy demand of the heart. During adrenergic stimulation, contractile activity is stimulated, increasing the demand for energy. Normally, this increase in demand for energy is adequately met by adrenergic stimulation of fuel catabolism (e.g. through PKA and Ca^{2+}-dependent mechanisms), so adrenaline turns off AMPK to prevent over-stimulation of catabolism. However, the energy demand arising from more intense contractile activity is not adequately met by adrenergic stimulation of catabolism, and under these conditions the AMP/ATP ratio begins to rise. This increase in the AMP/ATP ratio turns on AMPK. The present result shows that the inhibitory effect of adrenaline on AMPK is markedly blunted when AMPK is activated by the AMP-pathway. Thus, under these conditions, AMPK and adrenergic stimulation synergistically stimulate catabolism to meet the extra demand for energy.

3.10 Conclusion

The original aim of the present study was to investigate the adrenergic signalling pathway responsible for the effect of adrenaline to inhibit palmitate-induced AMPK activation. The present results suggest that, in addition to activation of AMPK by palmitate, adrenaline may also inhibit activation of AMPK by sorbitol-induced hyperosmolarity. In contrast to the observation in perfused hearts, adrenaline also showed an apparently direct inhibitory effect on AMPK. Because the effect of palmitate to activate AMPK was lost during the present study, this direct effect of adrenaline on AMPK was investigated. The present data, particularly the observations that (1) the effect of adrenaline was independent of a detectable change in the AMP/ATP ratio, (2) the effect was mimicked by PMA, (3) adrenaline increased phosphorylation of the AMPK β1 subunit suggest the possibility of a novel signalling/mechanism which regulates AMPK. The results from the present study also highlight the tissue specificity of adrenergic signalling and AMPK regulation.

3.11 Suggestions for further study

1. Investigation of whether a change in NAD^+/NADH is responsible for the inhibitory effect of adrenaline on AMPK.
2. Investigation of the effect of adrenaline with electrically stimulated cardiac myocytes to determine if contraction is responsible for the lack of the effect of adrenaline in perfused hearts.
3. Characterisation of the inhibitory effect of adrenaline on AMPK in the presence of sorbitol to clarify if the inhibitory effect seen when AMPK is activated by sorbitol is mechanistically identical to the effect of adrenaline under basal conditions.
3. Investigation of the effect of hyperosmolarity on AMPK activation in perfused hearts and the effect of adrenaline on hyperosmolarity-induced AMPK activation.
4. Investigation of the effect of adrenaline on the AMP-independent activation of AMPK by hypoxia and metformin.
5. Investigation of the adrenoreceptor subtypes mediating the effect of adrenaline using selective agonists and antagonists.
6. Investigation of G proteins involved in the effect of adrenaline using CTX, PTX, and a G_q inhibitor.
7. Determination of the effect of selective PKC inhibitors on α_1 and β mediated effect.
8. AMPKK activity assay using recombinant AMPK with total cell lysates or CAMKKβ and LKB1 immunoprecipitated from cardiac myocytes treated with or without adrenaline.
9. Investigation of the effect of adrenaline on association of AMPK with AMPKKs.
10. Investigation of the effect of adrenaline on phosphorylation of the AMPK β2 subunit.
11. Identification of the site on β1 subunit phosphorylated by adrenaline and investigation of the role of this site in the effect of adrenaline by site directed mutagenesis.

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